2. Review of literature

2.1. Acute kidney injury: a global concern

AKI is defined as a gradual decline in the function of the kidneys, resultant of fluid imbalance, failure to maintain electrolyte and acid-base balance. AKI supersedes the previous term 'Acute Renal Failure' (ARF) and is a spectrum, ranging from a less severe form to an advanced form, which demands the urgent need of Renal Replacement Therapy (Kellum, 2016). AKI is a serious clinical condition that has sole and diverse pathophysiology. AKI has been classified into three forms including pre-renal AKI, intrinsic AKI, and post-renal obstructive nephropathy. Pre- and post-renal AKI are the outcomes of distant-renal disorders which cause a reduction in GFR, whereas, 'intrinsic' AKI considered as actual kidney injury. Moreover, the persistence of pre- and/or post-renal AKI would eventually be evolved to renal cellular injury and thus intrinsic AKI (Cardenas, 2013). In addition, AKI-induced multiorgan dysfunction is of prime concern which results in a higher mortality rate (Lee et al., 2018). Due to the organ crosstalk, intrinsic renal damage may cause injury to distant organs like the liver, heart, brain, bone marrow, lungs, and intestines. The cellular pathophysiology of AKI belongs to possible pathways like metabolic acidosis, electrolyte and water imbalances triggered life-threatening arrhythmias, ROS, vascular inflammation, overproduction of systemic cytokines, neurohumoral factors, proapoptotic pathways and foremost RAS pathway (Bucsics & Krones, 2017; Lee et al., 2018).

2.1.1. Epidemiology of acute kidney injury

The calamitous growth in the incidences of AKI, together with mortality and medical cost attributable to AKI precipitates a huge social, financial, and health system burden worldwide. An analysis of post-discharged patients who did not recover their renal function showed ~60% mortality within 1 year, which was thrice to fully recovered patients. The patients showed recovery within 7 days recorded only 10% mortality (Coorevits et al., 2013). An observational cohort study with collected data from 1988-2003 of cardiopulmonary bypass surgery patients showed that the elevation in age-sex-morbidity during the AKI study period from 1.1% to 4.1% (Swaminathan et al., 2007). In 2015, four

major clinical studies have conducted on sepsis, stroke and HIV-adults hospitalized AKI patients which showed increased incidences of dialysis requiring AKI from 5.2% to 6.6%, 15.9 to 208.7, 0.09% to 0.18% and 0.7% to 1.35%, respectively (Sawhney & Fraser, 2017). In 2013, the International Society of Nephrology (ISN) declare a statement; nobody should be dying of untreated AKI in low-income regions (like Africa, Latin America, and Asia) by 2025 (Yang, 2016). In a meta-analysis data pooled from hospitalized patients, revealed that the incidences of AKI were 19.4% in Eastern Asia, 16.7% in Western Asia, 31.0% in Southeastern Asia, 7.5% in Southern Asia and 9.0% in Central Asia (Susantitaphong et al., 2013). Additionally, the mortality percentage of the AKI population remained 36.9% in Eastern Asia, 23.6% in Western Asia, and 13.8% in Southern Asia (Susantitaphong et al., 2013). Therefore, the prevalence of AKI is similar among the countries irrespective of resources, despite renal replacement therapy is less frequently utilized in low-income based countries than the developed ones. In developing regions, AKI being considered as the consequence of pre-existing diseases, and thereby the diagnosis is, in general, delayed. Together, the identification and management of AKI need improvement, where unfulfilled needs like improved diagnostics and therapeutic strategies should be evolved to advance the care of AKI patients.

2.1.2. Clinical manifestations in acute kidney injury

Patients with AKI need a vigilant history, cautious interpretation of laboratory tests, and imaging, along with thorough physical examination (Kaushal & Shah, 2014). The clinical symptoms associated with AKI relay on the degree of renal functional injury. During the initial phase of AKI, the only clinical feature is renal dysfunction as measured by blood tests (BUN, Creatinine) and little cases of oliguria. Advance stages of AKI showed some severe clinical manifestations such as nausea, vomiting, platelet dysfunction, bleeding, declined immunity, pruritus, dyspnea, hypertension, cardiac insufficiency, pericarditis, pleuritis, tremors, anxiety, seizure and ultimately coma. These symptoms are the repercussion of reduced renal function provoked by expanded volume, hyperkalemia, metabolic acidosis, and accumulations of uremic toxins (Bockenkamp & Vyas, 2003; Bouchard et al., 2009; Evans & Greenberg, 2005). The identification of patients with pre-existing co-morbidities, including age, hypertension, diabetes, or vascular disorder, is crucial during the evaluation of patients alleged with AKI. Guidelines by Kidney Disease:

Improving Global Outcomes (KDIGO) group has given the diagnostic criteria for AKI in which serum creatinine (sCr) showed significant elevation within 48 h with or without oliguria. However, elevated sCr levels are not the true diagnostic feature, due to its association with the above-mentioned pathophysiologic mechanisms (McMahon & Koyner, 2016). Urine dipstick and microscopic tests are beneficial as they come up with clues on the cause of AKI. Urine samples showing the presence of crystals, cells like red cells, confirm glomerulonephritis (Pickering & Endre, 2014). Moreover, histological analysis of kidney samples taken from rat AKI models generally shows the presence of red blood cells in the lumen, dysmorphic red cells, and their cast formation signifying acute interstitial nephritis and abnormality in the epithelial cell structure (Cortes et al., 2018; Zhang et al., 2016).

2.1.3. Phases of acute kidney injury

AKI has been designated with different terms depending upon the affected part of kidney tissue and the main categories are pre-renal, intrinsic, and post-renal (Makris & Spanou, 2016). Amongst, intrinsic AKI is quite challenging to assess because four different structures (interstitium, tubules, glomeruli, kidney blood vessels) are involved in the injury. Acute tubular necrosis (ATN) is the term used to nominate AKI, results from damage to the tubular cells (Alobaidi et al., 2015). In clinical aspects, AKI is characterized by four phases: initiation, extension, maintenance, and recovery phases ***(Figure 2.1)***. The initiation phase is ushered by a drastic fall in renal blood flow followed by the grievous depletion of ATP levels. This leads to severe dysfunction of renal cells (Devarajan, 2006). These conditions are imitated by experimental IRI models, demonstrating structural and functional changes in tubular cells (Dube et al., 2017; Raup-Konsavage et al., 2018; Zuk & Bonventre, 2016). These alterations disrupt the normal functioning of renal epithelial and vascular cells. Cell necrosis in the early initiation phase leads to the release of cytokines and other inflammatory mediators (Lee et al., 2011; Lee et al., 2017b). The extension phase is characterized by sustained necroinflammation at the corticomedullary junction of the kidney. To compensate for function loss, the proximal tubular cells of the outer cortex enter endocycles to increase their functional capacity by undergoing polyploidization and hypertrophy (Lazzeri et al., 2018). Moreover, the glomerular filtration rate (GFR) used to gradually fall due to continuous corticomedullary injury (Basile et al., 2012). Therefore,

this phase could be considered as a better therapeutic target option for the treatment of AKI. The maintenance phase is sorted by cellular repair, migration, and programmed cell death along with progenitor proliferation in order to endeavor clonal regeneration of denudated tubular segments to regain tubular integrity (Bird & Walker, 2015; Lazzeri et al., 2018). This phase is also known as the reorganization phase in which due to tubular cell endocycle and progenitor-driven regeneration of necrotic S3 segments GFR recovers, although the extent to which recovery is possible depends the amount irreversibly lost nephrons in which regeneration failed. Therefore, irreversible nephron loss is common upon acute tubular necrosis and accounts for the high frequency of CKD after an AKI episode.

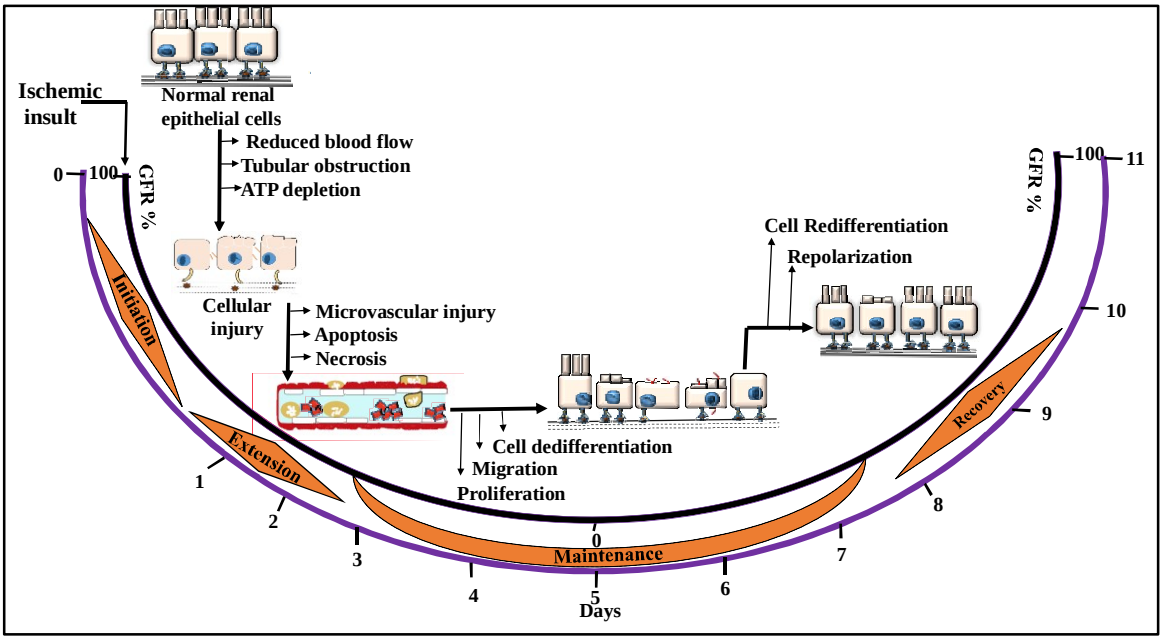

Figure 2.1 Link between clinical phases and cellular phases of AKI represented by alteration in GFR.

2.1.4. Effect of hyperglycemia on acute kidney injury

Diabetes is defined as a chronic condition whose incidences and prevalence have been rapidly increasing worldwide (Collaboration, 2016). One-third of such populations complained about chronic renal complications. Additionally, DM has also emerged as a major risk factor for renal hypoxia, supposedly linking AKI and hyperglycemia (Hertzberg et al., 2015; Takiyama & Haneda, 2014). Patschan and Muller have summarized that maintenance of blood glucose levels has significantly decreased the chance of AKI

episodes in ICU (Patschan & Müller, 2016). They further highlighted DM as a "fast-acting" risk factor for AKI susceptibility to ischemia. The chances of tissue vulnerability increase as a consequence of significant micro-vasculopathy and tubulointerstitial inflammation. The last-mentioned effects might occur in non-diabetic patients in whom acute hyperglycemia is not controlled (Patschan & Müller, 2016). However, treatment with insulin precipitated strict glycemic control and cause an inconsistent diminution of postoperative renal impairment, but rise the hypoglycemic complications. Current understandings support to control of the hyperglycemic stage by moderate glycemic levels (Mendez et al., 2016). Under cardiac complications such as coronary artery bypass grafting surgery (CABG) and acute myocardial infarction (AMI), acute hyperglycemia remains the predictor of AKI, predominantly in patients without diabetes. A study conducted on 307 patients undergoing CABG revealed the 48.2 % of such patients shown HbA1c $\geq$ 6.0 % along with the incidence of AKI (Oezkur et al., 2015). Recently, a cohort study of 474 diabetic patients with AMI observed the 16% of individuals develop AKI (Marenzi et al., 2018).

Apart from it, experimental studies have also supported the concept that hyperglycemia increased the risk for AKI as evidenced by the activation of several pathogenic pathways like mitochondrial ROS production (Peng et al., 2015), interleukins production(Sehnine et al., 2018) and apoptotic renal cell degeneration (Gong et al., 2019). Thus, collected clinical and experimental study data highlighted the need for further research to minimize the mortality rate associated with diabetes- AKI comorbidity.

2.2. Pathogenesis of acute kidney injury: Focusing on ischemia/reperfusion renal injury

2.2.1. Ischemia/reperfusion renal injury

I/R renal injury considered as the commonest cause of AKI (Bonventre & Yang, 2011) leading to localized loss of oxygen and nutrient supply to the cells, consequently the removal of waste products from the kidney (Le Dorze et al., 2009). There is an imbalance in the local tissue oxygen supply, need and accretion of waste products results in damage to tubular epithelial cells. The pathophysiology of I/R injury is very complex, including the activation of several pathological pathways like the release of ROS, activation of

leukocytes and neutrophils (Jang & Rabb, 2009), and inflammatory adhesion molecules, endothelial dysfunction (Sharfuddin & Molitoris, 2011). Thus, it becomes worthy to deeply understand the mechanisms of I/R in the development of AKI ***(Figure 2.2).***

During ischemia, a decrease in oxygen supply shifts the cells from aerobic to anaerobic metabolism, which results in a reduced adenosine triphosphate (ATP) production and augmented intracellular lactic acidosis. This is followed by the disruption in lysosomal membranes along with breakage in the cytoskeleton (Kako et al., 1988; Sugiyama et al., 1988). The inhibited activity of membrane-bound Na^+/K^+ ATPase increased the Na^+ ions and water, causes cellular edema. Besides, Ca^{2+} accumulation activates Ca^{2+} dependent proteases like calpains. Due to increased pH, calpains remain inactive during the ischemic period but they are injurious after reduced lactic acidosis production at reperfusion time. The Ca^{2+} overloaded mitochondria started ROS generation and opened the permeability transition pores (mPTP) after reperfusion (Edelstein et al., 1997). Throughout the ischemia period, very less ROS being produced in comparison to the whole I/R duration due to a decrease in cytochromes, xanthine oxidase, NO synthase as well as depleted nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Becker, 2004).

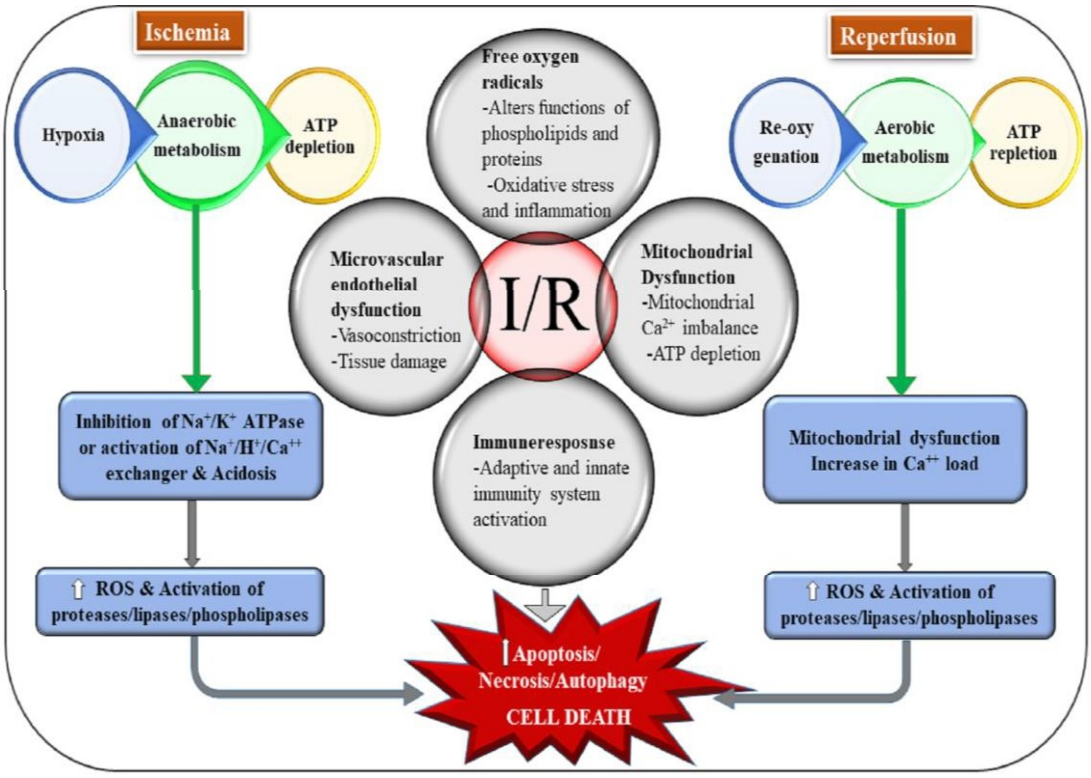

Figure 2.2 Pathophysiology of ischemia/reperfusion renal injury

At the time of reperfusion, the oxygen levels suddenly increase and get normalizes, which causes a deadly effect on ischemic cells. There is a sudden explosion of intracellular Ca^{2+} level and normalized pH activates calpains which cause severe injury to the kidney cells (Edelstein et al., 1997). Returning into normoxia results in the upsurge in plenty of ROS and the drastic reduction in antioxidant capacity (Martin et al., 2019), consequently dysregulation of electron transport chain (Chouchani et al., 2016). The combination of ROS and mitochondrial dysfunction (Ca^{2+} load), persuades apoptosis, and necrosis along with activation of the innate and adaptive immune system (Zhang et al., 2010).

2.2.2. Molecular mechanisms involved in ischemia/reperfusion renal injury

2.2.2.1. Role of oxidative stress in ischemia/reperfusion renal injury

Oxidative stress is the major contributor to the pathogenesis of AKI. On a cellular level, mitochondria remain the primary source of ROS production (Su et al., 2019). During the ischemic injury, there is an abrupt increment in ROS molecules like hydroxyl radical peroxynitrite and hyperchlorous acid. Simultaneously, the anti-oxidant enzymes, including superoxide dismutase (SOD), catalase, and glutathione reductase certainly declined (Dennis & Witting, 2017; Ratliff et al., 2016). IRI upregulates the level of proinflammatory cytokines and phagocytic recruitment, which also leads to ROS generation. Increased oxidative stress has an impact on vasoconstriction and renal vascular resistance (McCord, 1985). A study focused on renal ischemic injury defines the role of Nrf2 in the transgenic mice model. They revealed that wild type mice showed an increase in Nrf2-regulated cell defense genes, which were absent in knockout (-/-) mice. The reduction in Nrf2 expression leads to drastic impairment of kidney function (Liu et al., 2009), consequently proved that oxidative stress has an important role in the pathogenesis IRI. Another study demonstrated the increase in pericytes activated adhesion protein-1, a key protein responsible for local H2O2 gradient production, ultimately stimulated the neutrophil infiltration (Tanaka et al., 2017). Heme-oxygenase (HO) is the prime member of oxidative stress-induced renal damage. Two different studies showed that HO-1 knockout (HO-1-/-) mice had augmented sensitivity to ischemic injury as evidenced by an elevated inflammatory response and redox imbalance, as compared to wild type mice (Piantadosi & Suliman, 2006; Tracz et al., 2007). TRPM2, a nonselective cation channel, specifically found in proximal tubular cells, gets

stimulated by intracellular calcium, oxidative stress, ADP-ribose and TNF-α activity. Gao et al. performed a transgenic study using transient, receptor potential melastatin 2 (TRPM2) deficient mice (Gao et al., 2014). The deficient mice showed resistance to oxidants, NADPH oxidases (NOX) activity and apoptosis, exerted protective effect against IRI.

2.2.2.2. Role of inflammation in ischemia/reperfusion renal injury

A big body of evidence shows that the inflammatory response has a critical role in ischemic AKI. The inflammatory cascades that are activated by endothelial dysfunction might be augmented by the generation of a number of potent mediators in proximal tubular cells, representing a "maladaptive response" (Bonventre & Yang, 2011). These comprise proinflammatory cytokines i.e., TNF-α, IL-1, IL-6, TGF-β, and chemotactic cytokines i.e., MCP-1, IL-8, RANTES. Clinical studies demonstrated that the plasma levels of IL-6 and IL-8 predict mortality in AKI patients, while the urinary levels of CXCR3-binding chemokines help in predicting biomarkers for AKI patients after kidney transplantation (Vaidya et al., 2008). The complement system and toll-like receptor 2 (TLR2) may represent a vital component of the proinflammatory response in ischemic AKI (Amura et al., 2012). This study showed that TLR-2 and TLR-4 signaling induce the production of inflammatory cytokines (KC, IL-6, and TNF-α) from ischemic tubular epithelial cells, *in vitro*. Further scientists utilized mice with targeted deletion of complement factor B (fB-/-) or mice with targeted deletion of factor B and TLR-2 (fB-/-TLR2-/-). Surprisingly, they found that fB-/-TLR2-/- mice showed more severe injury compared to fB-/- mice, suggesting that blockade of complement system alone might be more protective than concomitant inhibition of complement system and TLR-2 in ischemic AKI. Bone morphogenic proteins (BMP) are a group of signaling mediators, belonging to the TGF-β superfamily of proteins. Two studies demonstrated the role of BMP-7 and BMP-5 in ischemic AKI. BMP-7 exerted a reno-protective role as evidenced by MCP-1, IL-8, IL-6, and IL-1 in cultured proximal tubular cells (Gould et al., 2002). Early endothelial outgrowth cells (eEOCs) have shown a protective effect in AKI and CKD. Patschan et al. utilized the concept and proved that BMP-5 promoted the renoprotection in ischemic AKI by activating eEOCs (Patschan et al., 2013). Morphologically, the inflammatory response cells like leukocytes revealed to get accumulate in proximal tubules, peritubular capillaries, and interstitial space of kidney after ischemic episode (Kinsey & Okusa, 2012).

Neutrophils remain the initial leukocytes to aggregate in the post-ischemic kidney. Interestingly, blockade of neutrophils or neutrophil depletion gives partly functional protection in experimental models (Han & Lee, 2019). Clinically, neutrophils are not the protruding marker of ischemic AKI, thereby developing qualms regarding its clinical importance. Then, scientists have recognized macrophages to get accumulate in ischemic animal models, in response to the recruitment of MCP-1 in tubular cells. Along with it, increased expression of chemokine receptor-CCR2 controlled the generation of pro-inflammatory monocytes facilitating tubular injury (Li et al., 2008a). MCP-1 aggravated tubular injury also required the coordination of T cells which was evident by clinical as well as animal studies (Cao et al., 2015a). Furthermore, reports suggested that severe kidney ischemia encourages the short- and long-term T lymphocyte infiltration and cytokine/chemokine activation, leading to kidney morphological alterations (Ascon et al., 2009; Gandolfo et al., 2009). On the other hand, B cells has a potential role in the pathogenesis of AKI. Jang et al revealed that overexpression of B cells reduced the repair of post-ischemic kidneys, *in vitro* and *in vivo*. However, treatment with anti-CD126 antibody augmented tubular proliferation and declined tubular atrophy in the long-term post-ischemic phase (Jang et al., 2010).

The crosstalk between the complement system and inflammatory cascades in ischemic AKI has been recognized attention in past decades (McCullough et al., 2013). A complement system is a group of systemic and cell membrane-based proteins that interact with the molecules of innate and adaptive immunity to accomplish essential functions. Under ischemic AKI, experimental (Thurman et al., 2005) and human (Stein et al., 1985) studies revealed augmented expression of C3 along with tubular basement membrane after ischemic insult. Recently, one study investigated the role of human C1-inhibitor (C1INH) targeting the complement system, in early and late phases of ischemic AKI (Danobeitia et al., 2017). The anti-inflammatory effect of C1INH was supported by declined markers of fibrosis, such as alpha-smooth muscle actin, desmin, and TGF-β1, concluding it as the effective therapy to prevent fibrosis after ischemic AKI. Further, the complement system has been detected to regulate pericyte-to-myofibroblast trans-differentiation (PMT) under the early phase of ischemic AKI in the swine model (Castellano et al., 2018). PMT was linked with a substantial reduction in peritubular capillary luminal diameter. Treatment

with C1-INH significantly protected the phenotype of pericytes preserving microvascular density and capillary lumen area in the tubulointerstitial. *In vitro*, the C5a transdifferentiated human pericytes showed PMT, regulated by extracellular signal-regulated kinases phosphorylation. It further, enhance the collagen I essential for both non-canonical and canonical TGF-β pathways. This study concluded the PMT as pivotal target and C1-INH as the potential therapy for the treatment of ischemic AKI. Upregulated complement system showed deteriorated renal functions and augmented intra-renal levels of IL-1β and TNF-α in unilateral nephrectomised AKI model of rat. Treatment with human mesenchymal stromal cells (MSCs) significantly improved the renal functions and reduced the inflammatory signaling molecules (IL-1β and TNF-α) (Zilberman-Itskovich et al., 2019).

2.2.2.3. Role of apoptosis in ischemia/reperfusion renal injury

Apoptosis is an extremely regulated and controlled process in which activation of the caspase signaling cascade leads to self-limiting programmed cell death. Caspases belong to the family of proteases and are of two types, including initiator caspases (-2,8,9,10) and effector caspases (-3,6,7) (Rai et al., 2005). The initiator caspases make a complex with apoptosome to get activated, which further activates effector caspases via proteolytic cleavage (Elmore, 2007). Apoptosis induced apoptotic bodies, possess intracellular proteolytic fragments, developed by the process called membrane blebbing. The apoptotic pathways could be initiated by the intrinsic pathway i.e. mitochondrial-dependent pathway where the first signal generated within the cell. Secondly, the extrinsic pathway i.e. cell death receptor pathway, where signals came out of the cell, involving TNF-α, first apoptosis signal (Fas)-ligand, FasL (Elmore, 2007). Interestingly, apoptosis is strongly regulated by the B-cell lymphoma 2 (BcL-2) family, which functions as protectors and inhibit apoptosis (BcL-2, Bcl-xL). BcL-2 family members might act as protectors (BcL-2, Bcl-xL) which inhibit apoptosis; sensors (BH3 only proteins, Bad, Bim, Bid) which inhibit protectors; and effectors (Bax, Bad) which initiate apoptotic signaling via enhancing the permeability of the mitochondrial membrane (Reed et al., 1996). Under intrinsic signaling, the upregulated effectors level enhances the permeability of the mitochondrial membrane leads to leakage of pro-apoptotic proteins. It is further accompanied by the caspase activator complex (apoptosome) formation (Nieuwenhuijs-Moeke et al., 2020). This

apoptosome split inactive procaspase-9 to active caspase-9, which further activates caspase-3. Under extrinsic signaling, TNF-α binds to its receptor and forms a complex called death-inducing signaling complex (DISC), consequently activates procaspase-8 and 10 (Locksley et al., 2001). The intrinsic and extrinsic pathways activate the initiator caspase-8, subsequently activates other caspases like caspase-3.

2.3. Epigenetics in acute kidney injury

Epigenetic regulation comprises the covalent modifications of DNA and histone proteins as well as RNA interference by non-coding RNAs in order to modulate gene expression. Covalent modification of DNA is achieved by DNA methylation, however, histone modifications carried out by numerous specific enzymes, also called epigenetic writers. All these modifications are recognized by epigenetic readers and might be removed by epigenetic erasers (Guo et al., 2019). Despite the pathogenesis of AKI has been unearthed on many levels, the epigenetic research of AKI has gained attention in recent two decades. Zager and his group demonstrated that epigenetic mechanisms are possibly be involved in all the developmental stages of AKI (Zager et al., 2006; Zager et al., 2007; Zager et al., 2005). It has been suggested that several epigenetic alterations linked with short- or long-term hypoxia associated with abrupt oxidative stress and persistent inflammation (Tang & Zhuang, 2019). As per the clinical data, the genetic predisposition (Vilander et al., 2015) alone is insufficient to explain the multifaceted pathogenesis of AKI (Mimura et al., 2016). ***Hence, this impulse-driven the focus of research towards epigenetic mechanisms involved in the development of AKI.*** The importance of epigenetics in the disease pathophysiology was not documented until the past two decades, where the abnormal DNA methylation could be linked to cancer development (Bird, 2002). Kidney development could be controlled by epigenetic modifications. Whereas, it has been well known that epigenetic modifications are being explored in the development and progression of many diseases, including AKI (Fan et al., 2013; Huang et al., 2015a; Liu et al., 2019a; Peng et al., 2015). ***Hence, the scientific approach towards epigenetic studies associated with AKI may help in the identification and development of novel therapies for the treatment and prevention of AKI progression.***

C. H. Waddington was the first scientist who defines epigenetic as "the causal interactions between genes and their products, which bring the phenotype into being'' (Waddington, 1942). With time, the definition modified as "***the heritable changes in gene expression patterns without any alteration in the underlying DNA sequences***" (Holliday, 1987). Epigenetic alterations involve a different type of histone post-translational modifications involving acetylation, methylation, phosphorylation, sumoylation, ubiquitinylation, carbonylation, and glycosylation, and DNA methylation (Dressler, 2008; Tang & Zhuang, 2015).

2.3.1. Histone Posttranslational Modifications

Chromatin is a complex figure of DNA and proteins present within the nuclei of eukaryotic cells. The fundamental repeating units of chromatin form the nucleosome, which is composed of positively charged histone protein cores (H2A, H2B, H3, and H4), wrapped around with 146 base pairs of negatively charged DNA (Chen et al., 2014c). Chromatin remodeling is a dynamic process in which chromatin might open or remain condense, leads to active or repressive gene transcription (Shen et al., 2000). The core histones are supposed to be globular excepting the amino-terminal ''tails'', which remain unstructured (Kornberg, 1974; Kouzarides, 2007). Regulation of gene expression depends on the approachability of DNA to several transcriptional factors, and coactivators/corepressors. Here, histone (PTMs) divide the genome into two distinct domains known as **euchromatin**, in which DNA is available for transcription, and **heterochromatin**, in which DNA is inaccessible for transcription (Kouzarides, 2007). Histone PTMs comprises of methylation, ubiquitination, glycosylation, ribosylation, biotinylation, phosphorylation, acetylation, citrullination, cis-trans isomerization on the N-terminal tail of the histones, that shape into a ***'histone code'*** (Tang & Zhuang, 2015). Chromatin modifying enzymes regulates these histone PTMs. For example, HDACs remove acetyl groups from histone proteins and participated in the growth and differentiation of the embryonic kidney (Chen et al., 2011). On the other hand, HMTs can add methyl groups to histone proteins and cause the aggregation or suppression of kidney diseases like AKI (Fontecha-Barriuso et al., 2018). In the following sub-headings, individual histone PTMs are being described.

2.3.1.1. Histone Acetylation

Histone acetylation is a type of histone modification that occurs when the acetyl moiety gets linked to the lysine residue of histone molecule in the presence of enzyme- histone acetyltransferases (HATs) (Zhuang, 2018). Histone acetylation is the most widely investigated histone modification, tends to counteract the positive charge of the lysine residue thereby facilitating the formation of euchromatin (or open chromatin) that in turn enables access to transcription factors which upregulates transcription (Zhuang, 2018). Like, the histone deacetylation process is facilitated by the presence of an enzyme- histone deacetylases (HDACs) (Fontecha-Barriuso et al., 2018; Gadhia et al., 2018). So far, only 18 mammalian HDAC proteins have been recognized till date, and known to have 4 classes categorized on the basis of domain organization and sequence homology with yeast orthologues: class I comprises HDACs 1, 2, 3 and 8; Class II comprises HDACs 4, 5, 6, 7, 9 and 10, class III contains sirtuin (SIRT) 1-7 and class IV comprises HDAC 11 (Fontecha-Barriuso et al., 2018). The classes I, II, and IV demand the presence of zinc for their catalytic activity, and hence they are regarded as 'classical' HDACs, however, class III HDACs depend on NAD+ for their catalytic activity (Fontecha-Barriuso et al., 2018; Zhuang, 2018). On the other hand, the most commonly recognized HATs members are-P300/CBP, MOZ-Ybf2/Sas3-Sas2-Tip60 (MYST), Gcn5-related N-acetyltransferases, nuclear hormone receptor co-activators and TATA-binding protein-associated factor 1 (TAF1) (Fontecha-Barriuso et al., 2018; Yuan & Marmorstein, 2013; Zhuang, 2018).

The process of acetylation tends to disturb the link between the negatively charged DNA and positively charged histone tail, whereas the HDACs flips these alterations. The HDACs also play a vital role in regulating the acetylation of more than 1750 non-histone proteins (Choudhary et al., 2009; Gadhia et al., 2018). Therefore, the HATs, HDACs, and their counterparts control the approach of large transcriptional molecules to chromosomal promoter sites which might result in either gene activation or gene silencing (Gregoretti et al., 2004). Histone acetylation has often been associated with various kidney diseases such as CKD associated with or without diabetic nephropathy, renal carcinoma, and AKI. The emphasis on the pathophysiological role of HATs and HDACs in AKI has been elaborated below.

2.3.1.2. Histone acetylation in acute kidney injury

H3 histone acetylation seems to increase the release of pro-inflammatory and pro-fibrotic mediators that may aggravate kidney injury (Zager et al., 2011). In the UUO model, total H3K9 acetylation (H3K9Ac) in the kidney was upregulated at day 10, defining the long term effect of AKI on H3K9 regulation (Hewitson et al., 2017). Whereas, IRI elevated the degree of histone acetylation in a gradual way in the kidney tissue. After day 21, the degree of histone H3 acetylation was found to be triple as compared to day1 of renal injury (Zager et al., 2011). In a severe unilateral IRI model, induction of transient decline in histone H3 acetylation in the proximal tubular region was observed. This caused a reduction in the activity of HAT. While during reperfusion, the acetylation in histone H3 was re-established and the production of bone morphogenetic protein-7 (BMP7) was initiated, BMP7 is one of the main regulators of renal repair, the decline in the HDAC5 may have contributed in the restoration of H3 acetylation (Zager et al., 2011).

Although contradicting studies have also been performed suggesting the increase in histone H3 acetylation occurred in moderate unilateral IRI after day 1 of reperfusion, the levels of H3 remained constant until 3 weeks (Zager et al., 2011). Interestingly, another study using bilateral IRI in mice model suggested an initial upregulation in the H3K14Ac followed by a decline to the basal levels after an interval (Havasi et al., 2013) ***(Table 2.1)***. In contrast, H4K5Ac and H4K12Ac were initially decreased but returned to basal levels after some duration (Havasi et al., 2013). It was observed that the dynamic regulation of the H4-specific HAT-JADE1 complex is responsible for the alterations in histone H4 acetylation (Havasi et al., 2013). There may be diverse justifications for the discrepancies among different studies such as variances in the severity of the injury, the strain of the animal used, time-points variations, and the different techniques utilized to evaluate histone acetylation.

Role of HDAC inhibitors in acute kidney injury

Under AKI settings, reno-protective genes like Klotho and PGC1a get significantly reduced, where the treatment with HDAC inhibitors averted their reduction in cell culture of tubular cells which were subjected to inflammatory cytokines (TWEAK, TNF-α) (Moreno et al., 2011; Ruiz-Andres et al., 2016b). In AKI, augmented levels of histone acetylation are often heterogeneous, which might attribute to the type of injury, time

duration, and specificity of a gene (Mar et al., 2015)**.** When the experimental rats were subjected to cisplatin-induced AKI, it induced class III HDAC protein: SIRT1, which appeared after 6 h of the cisplatin insult and was linked to histone H3 deacetylation. Nevertheless, the cisplatin-induced AKI did not alter the expression of HDAC5 and HDAC3 till day 2 and day 3, respectively (Sakao et al., 2011). Interestingly, the overexpression of SIRT1 showed a protective effect by preserving the peroxisome activity (Hasegawa et al., 2010). This study was supported by the findings of suppressed SIRT1 expression which decreased the resistance to oxidative stress in renal medullary cells. In the UUO mice model, the deficiency in SIRT1 expressions was found to exacerbate renal apoptotic and fibrotic signaling cascades (Sakao et al., 2011).

It has been observed that IRI downregulated the expression of HDAC1 without influencing the HDAC2 production in the ischemic kidney (Kim et al., 2013). In the initial phase of post-IRI, pre-treatment with pan-HDAC inhibitor, Trichostatin A (TSA) significantly ameliorated the function of kidney in the unilateral IRI model (Levine et al., 2015). MS-275 (class I HDAC inhibitor) significantly reduced renal fibrosis but showed lesser protection to kidney function at the early stage of AKI (Levine et al., 2015). In addition, knockout mice model of the HDAC6 gene has not shown any reduction in renal IRI (Levine et al., 2015). Interestingly, the administration of HDAC6 inhibitor (Tubastatin A) tends to protect against rhabdomyolysis-induced kidney as evidenced by reduced oxidative stress and inflammatory signaling pathways, indicating the role of HDAC6 differs with the type of AKI insult (Shi et al., 2017). Two well-known esterified derivatives of HDAC inhibitor; 4-(phenylthio) butanoic acid (PTBA), 4-methyl-thiobutanate (M4PTB) supported the repair of injured kidney tubules, intensified proliferation, reduced G_2/M arrest and ameliorated renal fibrosis, this was observed in mice model of severe IRI-AKI after 24 h post-ischemic insult (Cosentino et al., 2013).

Another study showed that mouse kidney exposed to IRI caused a momentary decline in histone acetylation during the ischemic injury, and in the proximal tubular cells it was seen that there was a decrease in the levels of isoenzyme HDAC5 amidst the recovery phase. Simultaneously, this caused histone re-acetylation and induced the expression of BMP7 in the proximal tubular region (Marumo et al., 2008). The expression of BMP7 plays a vital role in the nephrogenesis during kidney development and the recovery phase of ischemia

in order to regenerate and repair the tubular epithelial cells after IRI (Tomita et al., 2013; Villanueva et al., 2006). The decrease in the HDAC5 levels caused the return of BMP7 in the proximal tubular activator inhibitor type-1 (PAI-1), considered as a potential target in the treatment of IRI-aggravated inflammatory signaling and kidney dysfunction (Tomita et al., 2013). According to other studies, HDAC1 effectively binds to the promoter site of PAI-1 by implementing the chromatin immunoprecipitation (CHIP) assay, the binding occurred due to the IRI induction causing a negative regulation of PAI-1 gene expression. Hence, this mechanism offered a novel epigenetic target for AKI (Kim et al., 2013). In cultured renal epithelial cells, HBO1 is the enzyme which found to acetylate the histone in the presence of a protein known as the gene for apoptosis and Differentiation-1 (JASE1) (Havasi et al., 2013). The administration of paclitaxel (Taxol), a microtubules stabilizer that facilitates tubulin acetylation, controls tubular cell proliferation, aids in the recovery of impaired renal function and intensified the fibrosis during the recovery phase of renal IRI (Han et al., 2016).

In AKI, proximal tubular cells got highly influenced by endotoxin like lipopolysaccharide, LPS, and exaggerated the renal TNF-α levels. After IRI, LPS exposure enriched the TNF-α gene promotor site with the recruitment of RNA polymerase II (Pol II), which augment the dephosphorylated of histone H4S1ph and Pol II C-terminal domain (CTD) phospho-serine 2 levels. Yet, only IRI augmented the transcription-permissive histone phosphorylation (H3S10ph, H3S31ph) at the TNF-α gene promoter site. Interestingly, simultaneous activation of histone phosphorylation and acetylation regulated the gene expression. IRI and LPS augmented acetylation of histone H3 like H4K5/8/12/16Ac, H2KA5Ac, H3K9/14Ac, H2BK4/7Ac. This study clarifies the differential action of IRI and LPS on histone phosphorylation and acetylation regulating TNF-α gene (Bomsztyk et al., 2013). The therapeutic efficacy of histone acetylating/deacetylating agents often is dependent on the specific molecule and their dosage, the time of administration, and etiology behind the kidney injury. According to experimental renal injury reports, the class I and II HDAC inhibitors effectively prevent kidney damage, whereas these results varied with class I HDAC inhibitors (Brilli et al., 2013).

The influence of HDAC inhibitors on AKI was initially studied in cisplatin-induced nephrotoxicity, they found conflicting results (Arany et al., 2008; Dong et al., 2008). It was

reported that TSA and suberoylanilide hydroxamic acid (SAHA) showed toxicity against cultured renal tubular cells (Dong et al., 2008), while another study observed that TSA exhibited cytoprotective effect against cisplatin-induced nephrotoxicity in cultured renal tubular cells (Arany et al., 2008). A supplementary study was performed to assess the activity of TSA and SAHA on renal tubular cells, it was found that both drugs exerted renoprotective effect against cisplatin-induced tubular cell apoptosis by re-establishing CREB-mediated transcription, dose-dependently (Dong et al., 2010). Pang et al. have reported that TSA significantly attenuated the renal tubular apoptosis and decreased caspase-3 activity and along with renal fibrosis in the UUO-AKI as evidenced by decreasing STAT activation, and enhancing H3 and H4 acetylation (Pang et al., 2009). Likewise, TSA exhibited a reno-protective effect against unilateral IRI via increasing H3 acetylation and microRNA-21 expression (Levine et al., 2015). Additionally, SAHA showed the reno-protective activity by preventing renal tubular apoptosis induced by hemorrhagic shock in the rat model, it is often linked to the upregulation of H3K9Ac expressions and decreased the BCL-2 associated death promoter protein (BAD) expression (Zacharias et al., 2011). Whereas, valproic acid (VPA), a non-selective HDAC inhibitor, reported to reduce the protein excretion and kidney damage in Adriamycin-induced and IRI induced AKI models (Van Beneden et al., 2011; Zacharias et al., 2011).

Subsequently, VPA improved the kidney function tests, suppressed apoptotic and inflammatory signaling in the ischemic kidney, following increased Klotho expression by inhibiting TWEAK signaling (Costalonga et al., 2016). Pre-treatment with Entinostat, class I HDAC inhibitor reported to protect the kidney against IRI, by maintaining the blood urea nitrogen levels for a longer time, but the magnitude was lesser than TSA. Nonetheless, MS-275 found to exaggerate the renal injury in folic acid-induced AKI or rhabdomyolysis, as demonstrated by increased apoptosis (Tang et al., 2014). The justification for the inconsistency between the protective effect in ischemic kidney and enhanced injury in nephrotoxic-AKI is still ambiguous. Besides, the genetic difference may be present amongst the function of Class I HDAC inhibitors for various types of AKI. Continuous administration of Entinostat in the nephrotoxicity studies till 48 h before the sacrifice could hinder kidney regeneration, this was verified by the decline in epidermal growth factor receptor (EGFR) expression and phosphorylation, it also decreased the expression of PAX2

and proliferating cell nuclear antigen (PCNA) (He et al., 2013; Tang et al., 2014). In UUO and aristolochic acid-induced AKI, treatment with Entinostat and M4PTB significantly prevented the renal fibrosis (Liu et al., 2013b; Novitskaya et al., 2014). In addition, MS-275, a HDAC1, and HDAC3 inhibitor reduced the renal injury as shown by an increased anti-inflammatory protein known as activated microglia/macrophage WAP domain protein (AMWAP) in cisplatin-induced and IRI-AKI (Levine et al., 2015; Ranganathan et al., 2016). Other studies involving different HDAC inhibitor called 4-methyl-thiobutanate (m4PTB) was studied in mouse models of AKI. It was found that m4PTB augmented proliferation and reduced the G2/M arrest of renal proximal tubules, thereby providing a reno-protective effect against cisplatin-induced AKI (Ranganathan et al., 2016).

Various experiments have also been carried out to assess the effect of curcumin, a HAT: p300/CBP associated factor (PCAF) inhibitor in the rodent models of cisplatin-induced and IRI AKI (Bayrak et al., 2008; Kuhad et al., 2007). It was observed that curcumin administration exhibited renoprotective effects as indicated by suppressed inflammatory and oxidative stress signaling and downregulated PCAF expression against rat model of cisplatin nephrotoxicity (Kuhad et al., 2007). Generally, HDAC inhibition tends to shield the kidney from several injuries and support repair processes ***(Table 2.2)***. The studies describing the utility of specific HDAC isoforms in AKI and their development are still impending.

Table 2.1 Epigenetic alterations in acute kidney injury

Histone modification	**Type of AKI**	**Findings**	**References**
Histone acetylation	I/R	Interacts with JADE1S-HBO1complex	(Havasi et al., 2013)
	I/R	Triggers histone acetylation which interacts at the TNF-α gene to produce endotoxin hyper-response in AKI	(Bomsztyk et al., 2013)

	Folic acid	Control the activation of EGFR/STAT3 and EGFR/Akt pathway during AKI	(Tang et al., 2014)
	I/R	Activates BMP7 in proximal tubular cells	(Marumo et al., 2008)
Histone Methylation	Maleate Endotoxin UUO	↑H3K4Me3 at Tnf-α, MCP-1, HMGCR genes	(Naito et al., 2008; Naito et al., 2009a; Naito et al., 2009b)
	Azotemia	↑H3K4Me3 at MCP-1 gene	(Munshi et al., 2011)
	I/R	↑H3K4Me2, ↑TGF-β1	(Chen et al., 2015)
Histone Phosphorylation	I/R	↑ Phosphorylation of H2AX positive tubular epithelial cells and ↑ DNA damage response	(Kim & Padanilam, 2015; Ma et al., 2014)
Histone Crotonylation	Folic acid	↑ enrichment of histone crotonylation at the PGC-1α and the sirtuin-3 decrotonylase genes, and proved histone crotonylation as reno-protective.	(Chen et al., 2018; Ruiz-Andres et al., 2016a)
	Hemodialysis patients	↓ histone crotonylation in patients with kidney failure reduced hemodialysis complications	

Note: *I/R- Ischemia/reperfusion; BMP-7- bone morphogenetic protein-7**;** Tnf-α- Tumor necrosis factor-alpha; MCP-1- Monocyte chemoattractant protein-1; HMGCR- 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; PGC-1α- Peroxisome proliferator-activated receptor-gamma coactivator (*PGC*)*-1alpha.

2.3.1.3. Histone methylation

The addition of a methyl group from a methyl donor S-adenosyl methionine (SAM) to a lysine or arginine residue of core histones, is known as histone methylation. It occurs on either of the three basic amino acids, preferably it happens on lysine and arginine sites but seldom at histidine sites (Greer & Shi, 2012). Lysine residue can get monomethylated, dimethylated, or trimethylated (me1, me2, or me3) on its ε-amine group, whereas arginine could be monomethylated and demethylated (me1 or me2), on its guanidyl group (Greer & Shi, 2012). Histone methylation could promote the active or repressive gene transcription, relatively based on the numeral methyl groups or kind of amino acids methylated. Histone H3 methylation at lysine 4 (H3K4), H3K36 and H3K79 are linked with active gene transcription, whereas, the addition of methyl group at lysine 9, 27 and 20 (H3K9, H3K27 and H4K20) has been linked to suppression in the gene transcription (Sims III et al., 2003). For a long time, it was believed the histone methylation is irreversible, but the discovery of histone demethylases like lysine-specific demethylases involving LSD1 and LSD2, and Jumonji (Jmj C)-domain containing proteins offered a challenging thought (Kooistra & Helin, 2012).

2.3.1.4. Histone methylation in acute kidney injury

In experimental AKI studies, I/R, maleate, endotoxin, and UUO mice models have reported increasing H3K4Me2 levels in corresponding to TNF-alpha, MCP-1 and HMG-CoA reductase genes in mouse models of AKI (Naito et al., 2008; Naito et al., 2009a; Naito et al., 2009b). In clinical settings, Munshi et al. examined the chromatin shed in the urine samples of azotemia patients and reported the enhanced H3K4me3 mark at the MCP-1 promoter-specific gene (Munshi et al., 2011). During I/R injury, hypoxia instigated vascular insufficiency which may lead to TGF-β1 synthesis (Jang et al., 2013). Chen et al. have reported the role of TGF-β, inflammatory mediators, as well as apoptosis in the progression of I/R, induced AKI (Chen et al., 2015). IRI induced renal dysfunction causes disturbed tubular morphology, renal cell apoptosis, and inflammation. Treatment with Apelin, a bioactive peptide, significantly reduced the Tgf-β1 expression, inflammation and renal cell death, subsequently improved renal tubular morphology. Besides, apelin effectively inhibit the elevation of histone methylation (Chen et al., 2015) ***(Table 2.2)***. It

also inhibits Kmt2d, HMT specific for H3K4me2, resulting renal I/R injury. Moreover, these results were also supported by *in vitro* study.

Enhancer of Zeste Homolog 2 (EZH2), HMT specific for H3K27Me reported being crucial in AKI and renal fibrosis (Zhou et al., 2016). In folic acid and ischemia-induced AKI, increased EZH2 levels worsen the renal function and tubular injury. Treatment with 3-deazaneplanocin A (3-DZNeP), a specific EZH2 inhibitor, significantly protects against renal tubular cell injury and death (Zhuang, 2018). In the long term, AKI is often associated with CKD. In the UUO injured mice model, EZH2 contributes to developing renal fibrosis along with vimentin, a mesenchymal marker, and reduced expression of E-cadherin (Zhou et al., 2016). These pathological alterations were significantly attenuated with pharmacological inhibition of EZH2 with 3-DZNeP. These studies proved the protective effect of 3-DZNeP in AKI and its progression to CKD.

2.3.1.5. Histone methyltransferases in acute kidney injury

A) Role of G9a in acute kidney injury

G9a is a well-known HMT which catalyzes the addition of either single or two methyl groups at H3K9 (me1 and me2), a repressive chromatin marker (Tachibana et al., 2001; Tachibana et al., 2002). In the developmental stage, G9a is primarily expressed in the Pax2+ cap mesenchyme, the proximal and distal tubules (McLaughlin et al., 2014). A number of studies confirmed that G9a is upregulated in several human tumors, such as colorectal cancer, breast cancer, bladder, and ovarian cancer (Chen et al., 2017; Huang et al., 2010; Zhang et al., 2015). (Dong et al., 2012) has revealed that TGF-β1 served as a G9a inducer involved in the EMT process inside the cancerous cells. As TGF-β1 is a major cytokine in tissue fibrosis, (Irifuku et al., 2016) hypothesized the link of G9a and TGF-β1 in the development of UUO-induced renal fibrosis. Interestingly, they found elevated G9a levels in mice kidney of UUO and human kidney from IgA nephropathy patients. Administration of G9a siRNA and BIX01294 (diazepin-quinazolin-amine derivative), a specific inhibitor of G9a, inhibited TGF-β1-induced ECM deposition and renal fibrotic changes, supported by increased and decreased klotho expressions and methylation of H3K9Me1. Similar results were obtained by *in vitro* culture of renal epithelial cells. In addition, pharmacological inhibition of G9a protected hair cells aminoglycoside-induced damage in the AKI mice model, signifying a potential role of G9a in the pathogenesis of

AKI (Yu et al., 2013). As data on AKI is very limited, thereby, further investigations are needed.

In the developmental process of pharmacological inhibitors, numerous small molecule G9a inhibitors have been established to counter the catalytic activity of G9a through various cell-based and animal-based experiments. As discussed above, BIX01294 remains one of the first molecules introduced as the competitive inhibitor of G9a. BIX01294 only competes with the G9a substrate and left the G9a cofactor *S*-adenosyl-methionine (SAM), the source of the transferred methyl group. After all, the intrinsic toxic effect of BIX-01294 instigated the development of another G9a inhibitor i.e. UNC0638. It is highly potent and specific to G9a with lesser cellular toxicity. UNC0638 exerted anti-cancer activity in various cancer cell lines, however, it has poor pharmacokinetics (Zhou et al., 2018). Further, UNC0642 was developed with better pharmacokinetics and high selectivity for G9a (Liu et al., 2013a). To date, the effect of UNC0638 and UNC0642 has not been evaluated in renal disease, including AKI.

B) Role of Enhancer of Zeste 2 in acute kidney injury

EZH2 is the HMT having functional enzymatic component of polycomb repressor complex 2 (PRC2), catalyzes the trimethylation of histone H3 lysine 27 (H3K27Me3). Whole PRC2, involving EZH2 is essential for regulating numerous genes required for the development and tumorigenesis. In the renal tissue, EZH2 transcripts are identifiable throughout embryonic development, but not detectable in adult kidneys (Caretti et al., 2004). At different stages of development, localization of EZH2 protein within the cells showed its different regulatory role at initial and later periods of organogenesis. It has been reported that EZH2 is required for both cellular proliferation and differentiation during organogenesis (Martinez & Cavalli, 2006; Oktaba et al., 2008). A growing body of evidence directed that unusual expression of EZH2 is linked with the development of CKD. In the UUO murine model of renal fibrosis, EZH2 was found in myofibroblasts and damaged renal epithelial cells. The kidney biopsy samples showed an elevated level of EZH2, collected from focal segmental glomerulosclerosis and IgA nephropathy patients (Zhou et al., 2016). Further, downregulation of EZH2 by inhibitors or siRNA in the renal cells has declined the activation of renal interstitial fibroblasts and epithelial-to-mesenchymal transition (*EMT*) of renal tubular cells. Similarly, the EZH2 inhibitor 3-

deazaneplanocin A (3-DZNeP) and GSK126 diminished the UUO-induced renal fibroblast activation along with partial inhibition of EMT and fibrosis in mice (Zhou et al., 2018; Zhou et al., 2016). The antifibrotic actions of EZH2 inhibition were exerted by dephosphorylation of multiple profibrotic receptors, such as epidermal growth factor receptor and platelet-derived growth factor receptor, followed by suppression of various intracellular pathways, like extracellular signal-regulated kinase ½ (ERK1/2), Smad3, signal transducer and activator of transcription 3 (STAT3) and AKT pathways (Wang et al., 2005; Zhou et al., 2016). These analyses suggested that EZH2 triggers the activation of renal fibroblasts, eventually renal fibrosis. One conflicting report claimed that increased levels of EZH2 protected the diabetic podocytes against oxidative stress and renal injury, as evidenced by triggered podocyte injury via pharmacologic or genetic depletion of EZH2 (Siddiqi et al., 2016). These findings were further supported by further studies performed with murine models of nephrotoxicity and subtotal nephrectomy (Majumder et al., 2018). The diverse functions of EZH2 in podocytes and renal tubules are unsettled but may show the differences in location and targets in the kidney.

EZH2 also contributes to the pathophysiological role of AKI. Recently, studies conducted on mouse models of I/R or folic acid-induced AKI revealed the renal tubules showed elevated expression of EZH2 and H3K27Me3, however; the pharmacological inhibition of EZH2 reduces the pathological alteration of renal tubules and attenuated the cell death. Moreover, *in vitro* inhibition of EZH2 via 3-deazaneplanocin A (3-DZNep) or knockout with siRNA resulting in the survival of renal epithelial cells from oxidative injury (Zhou et al., 2018). In contrast to it, Liang et al. demonstrated that administration of EZH2 inhibitor i.e. 3-DZNep after I/R injury attenuated the tubular injury and improved renal function as indicated by depleted cytokine production, decreased p38 levels (Liang et al., 2019). Besides, Li et al. demonstrated that rats treated with 3-DZNep resulted in attenuation of early acute renal allograft rejection via the reduction in the T cells activation and consequently the production of inflammatory cytokines (Li et al., 2016a). Apart from it, inhibition of EZH2 with 3-DZNep showed a renoprotective effect in cisplatin-induced AKI, as proved by the activation of E-cadherin (Ni et al., 2019).

The abovementioned studies clarify that EZH2 has a detrimental role in the renal tubules but a beneficial role in podocytes. Thus, targeting EZH2 may serve as a potential approach

for treating AKI, renal fibrosis, acute allograft rejection after transplantation, but not DN. Highlighting that EZH2 shields podocytes from injury, the use of EZH2 inhibitors should be avoided in podocytes focused studies.

It is worth noting that EZH2 inhibitors such as EPZ-6438, GSK126, and CPI-1205 have to get into clinical trials (Trial number: NCT01897571, NCT02601950, NCT02601937, NCT02395601, and NCT02082977). EPZ-6438 has shown remarkable effect against solid tumors and refractory B-cell non-Hodgkin lymphoma, proved it a great success as an anti-cancer therapy (Yan et al., 2016). Unfortunately, EZH2 inhibitors have not shown promising effects in human kidney diseases.

C) Role of SYMD2 in acute kidney injury

SYMD2, another HMT belongs to SET and MYND-containing lysine methyltransferases (SMYD). It can methylate both histone and non-histone proteins, including H3K4 and H3K36, and p53/TP53 and RB1 (Tracy et al., 2018). For the first time, elevated SYMD2 levels were identified in renal tumor tissue (Pires-Luís et al., 2015). In animal and human polycystic renal tubular epithelial cells from autosomal dominant polycystic kidneys showed a high expression of SYMD2 (Li et al., 2017). SYMD2 induced cystic renal epithelial cell proliferation was mediated by two pathways; STAT3 and NF-κB-p65. Pharmacological inhibition of SYMD2 by AZ505, a specific inhibitor of SMYD2, or using Pkd1-knockout mice (Li et al., 2017) revealed that SMYD2 exerted a crucial role in renal cyst growth. At present, the effect of SYMD2 in other kidney diseases (AKI, DN) remains to be elucidated.

D) Role of SUV39H1 in acute kidney injury

SUV39H1 is another HMT, catalyzes the trimethylation of H3K9, and associated with transcriptional repression of inflammatory genes. The high glucose condition, the effect of SUV39H1 on inflammatory gene promoters has very well reported in several cell lines, like vascular smooth muscle cells (VSMCs) (Villeneuve et al., 2008), macrophages (Li et al., 2016a) and cardiomyocytes (Yang et al., 2017b). A study conducted on clinical samples revealed the overexpression of SUV39H1 and H3K9me3 in the renal tubules of diabetic nephropathy patients, correlated SUV39H1 and H3K9me3 with the development of CKD (Wang et al., 2018). One of our studies demonstrated that hyperglycemia reduced the expression of SUV39H1 along with H3K9me3 and get related to renal fibrosis in type-

1 diabetes rats (Goru et al., 2016). Moreover, (Lin et al., 2016) have supported the findings of hyperglycemia-induced reduced expression of SUV39H1 and its associated HMT; H3K9Me3 in vascular smooth muscle cells and mesangial cells of diabetic mice. Remarkably, pharmacological or genetic inhibition phosphoinositide 3-kinase (PI3K) had suppressed SUV39H1 and H3K9Me3 of mesangial cells *in vitro*. Either genetic or pharmacological overexpression of SUV39H1 had protected the kidney tissue against the progression of DN (Lin et al., 2016). Collectively, these results indicate that alteration of SUV39H1 expression may promote the progression of DN, thus, targeting SUV39H1 may serve a novel therapeutic option for treating DN.

Numerous SUV39H1 modulators have been discovered and under experimental trials. In 2005, Chaetocin, a fungal mycotoxin isolated from Chaetomium minutum, has been established to be a specific inhibitor of SUV39H1(Greiner et al., 2005). But, further studies confirmed that chaetocin in higher concentration also inhibits G9a activity (Cherblanc et al., 2013), thereby showed non-specificity towards SUV39H1.

The clinical and experimental studies showed the potent cytotoxic effects of chaetocin in acute myeloid leukemia cells (Lai et al., 2015). It showed cardioprotective effect against myocardial infarction in a murine model of cardiac injury (Yang et al., 2017a) and brain injury associated with cerebral ischemia (Schweizer et al., 2015). Modulating SUV399H1 expression in distinct organs resulted in entirely different consequences. For example, the upregulation of SUV39H1 has successfully reversed the diabetic phenotype in the kidney of db/db models (Villeneuve et al., 2008). Whereas, cultured mouse mesangial cells treated with chaetocin augmented fibronectin and p21 (WAF1) protein levels (Li et al., 2016a). The abovementioned reports have clarified that SUV39H1 inhibition aggravates renal injury in the DN mouse model. To date, there is no data available for the effect of SUV39H1 modulation under other kidney diseases like AKI.

E) Role of Dot1/DOT1L in acute kidney injury

Methylation at H3K79 is catalyzed by two enzymes; Disruptor of telomeric silencing 1 (DOT1) and Dot1-like protein (DOT1L) present in lower eukaryotes and mammals, respectively (Min et al., 2003). They carried out the mono-, di-, and trimethylation at H3K79 which regulates the activation or repression of gene transcription (Min et al., 2003). The mono- and di-methylation of H3K79 contributes to gene transcriptional activation,

whereas H3K79 trimethylation involved with gene repression (Wong et al., 2015). DOT1L is the most studied HMT, exerting a potential role in many biological activities, like cell cycle progression, cardiomyocyte differentiation, somatic reprogramming, postembryonic growth, DNA damage feedback, and tumorigenesis (Zhou et al., 2018).

Administration of spironolactone resulted in reduced DOT1L expression and dysregulated endothelin-1 levels in proximal tubule specific in NRK-52 cell lines, demonstrating a plausible role of this HMT in the pathogenesis of diabetes (Wu et al., 2016). Additionally, another study showed spironolactone to ameliorate kidney injury by preserving DOT1L, which arbitrates downregulated endothelin-1 expression in type-1 diabetic rats (Zhou et al., 2012). In the murine model of UUO, DOT1L shown to be involved in the pathogenesis of renal fibrosis-associated CKD. Under *in vitro* and *in vivo* conditions, DOT1L and di-methylated H3K79 were highly expressed, which were effectively controlled by pharmacological inhibition of DOT1L with EPZ5676, consequently a reduction in renal fibrosis (Liu et al., 2019b). Another *in vitro* study showed that treatment with either EPZ5676 or DOT1L siRNA inhibits TGF-β1 induced fibrosis and EMT. Currently, the full knowledge of DOT1L in human kidney disease is elusive and needs further investigations.

F) Role of SET7/9 in acute kidney injury

SET7/9 is the protein methyltransferase which not only methylates histone proteins (H3K4) but also non-histone proteins and p53 (Kurash et al., 2008). Plenty of research carried out to elucidate the role of SET7/9 and neoplasms such as hepatocellular carcinoma, breast cancer, prostate cancer, and colorectal cancer (Wagner & Jung, 2012). In past decades, studies were conducted to establish the link of SET7/9 and renal diseases. Increased SET7/9 and H3K4me1/2/3 expressions have a critical role in the progression of renal fibrosis as evidenced by TGF-β1-regulation and overexpression of ECM associated genes (Sun et al., 2010). In db/db mice, kidney tissue showed enhanced recruitment of H3K4me1 and SET7/9 at MCP-1 promoters, indicating the potential role of SET7/9 in leukocyte infiltration (Chen et al., 2014a). However, siRNA treatment of SET7/9 notably reduced the MCP-1 expression and improved glomerular architecture, highlighting the potential role of SET7/9 in leukocyte infiltration via MCP-1 regulation in the kidneys of db/db mice. (Guo et al., 2016) showed that TGF-β1 mediated the recruitment of SET7/9 at the p21 gene promoter, despite siRNA treatment of SET7/9 significantly ameliorated TGF-β1-generated

p21 gene expression in rat mesangial cells. Moreover, SET7/9 regulates TGF-β1-mediated activation of renal fibroblasts via stimulating Smad-3 activation (Shuttleworth et al., 2018). In 2016, Sasaki et al have performed the research connecting cell line study to animals and to humans by utilizing Sinefungin, a specific inhibitor of SET7/9 demonstrated to attenuate renal fibrosis as specified by reduced expression of mesenchymal markers and ECM proteins in kidneys of UUO mice and cultured renal epithelial cells (NRK-52E and NRK-49F cells) (Sasaki et al., 2016). Interestingly, the augmented expression of SET7/9 was correlated with the level of interstitial fibrosis in kidney samples of patients with IgA and membranous nephropathy. TGF-β1 serves as a prime mediator of peritoneal fibrosis and apparently affects the expression of the SET7/9 in a methylglyoxal-induced murine model of peritoneal fibrosis and in non-adherent cells collected from the effluent of peritoneal dialysis patients. Administration of sinefungin attenuated the collagen deposition in the peritoneum as supported by diminished H3K4me1 (Tamura et al., 2018).

SET7/9 seems to play a critical role in the crosstalk of inflammation and hyperglycemia. In 2008, Li et al demonstrated the augmentation of inflammatory genes and SET7/9 recruitment in macrophages in diabetic mice. SET7/9 siRNA treatment significantly diminished TNF-α-mediated recruitment of NF-κB p65 at inflammatory gene promoters (Li et al., 2008b). Additionally, hyperglycemia augmented the expression of SET7/9 in glomerular fraction as well as the kidney of type1 diabetic rats (Goru et al., 2016). These reports suggest that the plausible role of SET7/9 in the progression of fibrotic diseases, and targeting SET7/9 with its specific inhibitors like sinefungin and Cyproheptadine (Takemoto et al., 2016), may serve as novel therapeutic strategies for fibrotic diseases, including diabetic nephropathy. However, lots of research has to be carried out to elucidate the role of SET7/9 in AKI.

G) Cyproheptadine: a novel histone methyltransferase-SET7/9 inhibitor

Structurally, Cyproheptadine comprises of *N*-methylpiperidine and tricyclic dibenzosuberene moieties, having molecular formula $C_{21}H_{21}N$ (Hirano et al., 2018). It is a potent H1 blocking antihistaminic and serotonin receptor blocker, which is clinically approved for the treatment of appetite stimulants in children (clinical trial: NCT01314989) (Krasaelap & Madani, 2017). Cyproheptadine has been tested against headache, migraine, depression, and neuroleptic akathisia (Fischel et al., 2001; Greenway et al., 1995; Okuma

et al., 2013). Moreover, Cyproheptadine has been evaluated against spinal cord injury based on its serotonergic receptor blockade activity (Murray et al., 2010). After spinal transection in rats, mRNA and protein levels of 5-HT2C receptors got altered in the absence of 5HT. The receptor activity maintained motoneuron excitability through regulating persistent calcium current, consequently results in muscle spasm. Notably, the administration of Cyproheptadine has significantly reduced muscle spasm of rats. Recently, Takemoto and his group have discovered Cyproheptadine as a novel scaffold of SET7/9 inhibitor (Takemoto et al., 2016). SET7/9 has shown crucial for the estrogen-dependent transactivation of Estrogen Receptor (ER) target genes. Further, treatment with Cyproheptadine effectively reduced estrogen receptor-α expression and transcriptional activity, consequently suppressed estrogen-dependent cell growth, subsequently helpful against breast cancer. Additionally, Hang et al have suggested that Cyproheptadine exerts an anti-nociceptive effect in cancer-induced bone pain via inhibiting SET7/9 and RANTES cytokine expressions (Hang et al., 2017).

Besides, Cyproheptadine contains dibenzosuberene in its structure. Based on this fact, (Hirano et al., 2018) started the synthesis of derivatives with a distinct functional group. Among all, 2-hydroxycyproheptadine seems to be more promising as it has a clear SET7/9 loop, thereby expected to be obliging for the development of novel SET7/9 inhibitors.

Table 2.2 Histone modifying agents in acute kidney injury models

Drug	**Type of AKI**	**Mechanism**	**Reference**
HDAC inhibitor			
TSA	Cisplatin-induced AKI	Exhibits cytoprotective activity against cisplatin-induced apoptosis in renal PT cells by re-establishing CREB-mediated transcription.	(Arany et al., 2008)
	UUO	Exhibits reno-protective activity: ↓ PT cell apoptosis, ↓ caspase-3 activation, ↓ STAT activation, and ↑ H3Ac and ↑ H4Ac.	(Pang et al., 2009)

	Unilateral IRI	Exhibits reno-protective activity in IRI by ↑ H3Ac and microRNA-21 expression.	(Levine et al., 2015)
SAHA	Hemorrhagic shock	Exhibits reno-protective activity by ↑ H3K9Ac and ↓ apoptosis and BAD expression.	(Zacharias et al., 2011)
VPA	Hemorrhagic shock	Exhibits reno-protective activity by ↑ H3K9Ac and BCL-2, ↓ apoptosis, and BAD expression.	(Zacharias et al., 2011)
	Adriamycin-induced nephropathy	Exhibits reno-protective activity by ↑ glomerular H3K9Ac expression.	(Van Beneden et al., 2011)
MS-275	Cisplatin-induced AKI	Exhibits reno-protective activity by ↑ anti-inflammatory protein AMWAP.	(Ranganathan et al., 2016)
	Unilateral IRI	Exhibits reno-protective activity in IRI by ↑ H3Ac	(Levine et al., 2015)
m4PTB	IRI	Exhibits reno-protective activity in IRI by ↑ proliferation and ↓G2/M arrest of renal PT cells.	(Cosentino et al., 2013)
HAT inhibitor			
Curcumin	Cisplatin-induced AKI	Exhibits reno-protective activity by ↓ inflammation, ↓ oxidative stress, and ↓ PCAF.	(Kuhad et al., 2007)
HMT inhibitor			
Apelin	Unilateral IRI	↓ Inflammation and TGF-β, tubular lesions, renal cell apoptosis in the progression of I/R induced AKI; inhibit H3K4me2 and Kmt2d, a histone methyltransferase.	(Chen et al., 2015)

3-DZNeP	IRI Folic acid-induced AKI	↓Renal tubular cell injury and death by attenuating EZH2 levels	(Zhuang, 2018)
	UUO	Reduced renal fibrosis along with vimentin, a mesenchymal marker, and reduced expression of E-cadherin; suppress the AKI to CKD transition.	(Zhou et al., 2016)

Note: *TGF-β-* Transforming growth factor-beta; AMWAP- *Activated microglia/ macrophage WAP domain protein; BAD-* BCL2 *associated agonist of cell death; BCL2- B-cell lymphoma 2; PCAF-P300/CBP-associated factor; TSA- trichostatin A; SAHA- suberoylanilide hydroxamic acid; VPA-Valproic acid; 3-DZNeP- 3-deazaneplanocin A; PT- Proximal tubules*

2.3.1.6. Histone phosphorylation

Another histone PTMs is 'histone phosphorylation' participates to changes the chromatin structure. The amino acid residues like serine, threonine, and tyrosine get phosphorylated on all four core histone proteins. Different protein kinases are required for the phosphorylation at distinct core histones, such as MEC1, the Aurora kinases and SPS1 (a sporulation-specific kinase) facilitating the addition of phosphoryl group in H2A, H3 and H4 of yeast, respectively. In mammals, mammalian sterile-20-like kinase (MST1) is the only protein kinase required for the H2B phosphorylation (Bhaumik et al., 2007). Histone phosphorylation credibly either euchromatin or heterochromatin depending on the position of amino acid modified, thereby, may lead to activation or repression of gene(s) expression. For instance, addition of phosphoryl group to histone H3 at serine 10 (H3S10ph), serine 28 (H3S28ph), threonine 11 (H3T11ph), tyrosine 41 (H3Y41ph) and phosphorylation of H2B at serine 32 (H2BS32ph) results in active gene transcription; however, addition of phosphoryl group in H2A at serine 1 (H2AS1ph), H2B at tyrosine 37 (H2BY37ph) and H4 at serine 1 (H4S1ph) leads to gene repression (Rossetto et al., 2012; Zhang et al., 2004).

2.3.1.7. Histone phosphorylation in acute kidney injury

Histone phosphorylation is said to be modulated by poly (ADP-)ribosylation. In 2001, Tikoo et al. utilized this concept in unearthing the link of histone phosphorylation and enhanced ROS production (Tikoo et al., 2001). The phosphorylation of histone H3

supported the heterochromatin and facilitated the injury and death of proximal tubular cells, which was proven by blocking poly-(ADP-ribose) polymerase (PARP) with 3-aminobenzamide or preventing H3 phosphorylation using selective MAPK inhibitor PD-98059. In various AKI studies, including cisplatin and renal IRI showed that the addition of phosphoryl group at histone H2A variant H2AX at serine 139 indicated as a marker of double-strand DNA damage (Ma et al., 2014; Pabla et al., 2008; Scholpa et al., 2014). Kim and Padanilam investigated the addition of phosphoryl group in histone H3 (p-H3), an indicator of the G2/M phase in the cell cycle, increased after ischemic insult, and supported by elevated levels of cyclinD1 (G2/M arrest indicator). It confirmed the role of p-H3 in cell cycle arrest mediated interstitial fibrosis after IRI (Kim & Padanilam, 2015) ***(Table 2.1)***.

2.3.1.8. Histone crotonylation

In histone crotonylation, crotonyl group supplied by crotonyl-coenzyme A get attached at lysine residues present on core histones, and the process is catalyzed by histone crotonyltransferases (Fontecha-Barriuso et al., 2018). Histone crotonylation is another histone PTMs, recently identified by high-sensitivity mass spectrometry techniques as a novel histone lysine acetylation (Tan et al., 2011). Even so, the functioning histone crotonylation differs from histone acetylation. In general, histone crotonylation regulates the active gene transcription; however the plausible mechanism of gene regulation is uncertain, and further studies are required to explore this histone PTM (Fellows et al., 2018; Tan et al., 2011).

2.3.1.9. Histone crotonylation in acute kidney injury

In folic acid and/or cisplatin-induced AKI, increase histone crotonylation showed a crucial role in kidney tissues. Further analysis specified that administration of crotonate in *in vitro* and *in vivo* condition, the expression of mitochondrial biogenesis regulator PGC1α, and SIRT3 was increased. This is the first to report to show the reno-protective role against AKI (Ruiz-Andres et al., 2016a). A 2018 clinical study demonstrated the role of crotonylation in the patients of kidney failure underwent maintenance hemodialysis patients (Chen et al., 2018) ***(Table 2.1)***. The high throughput mass spectrometry analysis revealed that a reduction in histone protein crotonylation was detected in hemodialysis

patients. Therefore, little research has been done on histone crotonylation and future studies are required to further explore its role in AKI pathogenesis.

2.3.1.10. Histone Ubiquitination

Ubiquitin is an evolutionarily conserved 76 amino acid protein that conjugated the substrate proteins for degradation via the ubiquitin-proteasome system (UPS) by the process of ubiquitination (Pickart, 2001). Primarily, ubiquitin is activated by an ATP-dependent reaction linking a ubiquitin-activating enzyme (E1), which catalyzes its conjugation via a thioester bond to a cysteine residue in a ubiquitin-conjugating enzyme (E2). In the final catalytic step, ubiquitin is moved from the E2 enzyme to a target lysine residue in a particular substrate protein by the action of a ubiquitin-protein isopeptide ligase (E3) (Hershko et al., 2000). Substrates can either be mono- or polyubiquitinated. Polyubiquitination involves 26S proteasome to target proteins for the degradation whereas monoubiquitination tags the substrate protein and imparts a signal for a particular function (Hershko et al., 2000). One well-defined example of this process is the monoubiquitination of histones H2A and H2B although ubiquitination of H2B is less abundant (1%-2%) when compared with ubiquitination of H2A (5%-15%) (Zhang, 2003). Like other modifications, histone ubiquitination is a reversible modification, therefore, the steady-state of it is determined by the accessibility of free ubiquitin and enzymatic activities intricate in adding or deleting the ubiquitin moiety. The addition of a ubiquitin moiety to a histone is governed by E1, E2, and E3 enzymes while the deletion of ubiquitin moiety involves the action of enzymes called deubiquitinases (DUBs) (Hershko et al., 2000).

H2AK119Ub is facilitated by the BMI-1/RING-1A protein found in the human polycomb complex and is responsible for transcriptional repression (Zhang, 2003). In contrast, H2BK120Ub is mediated by human RNF20/RNF40 and UbcH6 and is involved in active transcription (Cao et al., 2005; Zhu et al., 2005). E3 ligase, BMI1, encourages H2A ubiquitination and *Hox* gene silencing via regulating H2AK119Ub and methyltransferase EZH2 specific for H3K27Me (Cao et al., 2005). The overexpression of H2B specific E3 ligase, RNF20, augmented the levels of H3K4Me and H3K79Me and induced *Hox* gene expression. Additionally, inhibition of RNF20/40 complex reduced H2B mono-ubiquitination, H3K4, and H3K79 methylation, along with suppressed *Hox* gene expression (Zhu et al., 2005). Thus, a balance between active and repressive chromatin

marks is required to keep normal gene transcription, any changes in it might consequence in abnormal gene transcription may result in abnormal gene transcription and disease condition. Recent evidence has investigated the involvement of epigenetic mechanisms in various kidney diseases, involving DN.

Gao et al. stated the role of active mark H2BK120Ub and repressive chromatin mark H2AK119Ub in the regulating fibrotic genes like *Fibronectin* and *Tgfb1* in rat glomerular mesangial cells under hyperglycemic condition (Huang et al., 2015b). Another study revealed that decreased activity of *Usp22*, a de-ubiquitinase specific for H2A/H2B ubiquitination, proved to increase the fibrotic genes in rat mesangial cells under hyperglycemic condition (Gao et al., 2013). As per our previous reports, Goru et al. have established the crosstalk between histone methylation and histone ubiquitination in the progression of renal fibrosis under diabetic nephropathy (Goru et al., 2016). The study concluded that H2AK119Ub and H2BK120Ub regulate diabetic renal fibrosis by modifying active (H3K4Me2) and repressive (H3K9Me2) chromatin marks via controlling the expression of their respective HMTs, SET7/9, and SUV39H1. These reports clearly represented the role of histone ubiquitination in the progression of diabetic nephropathy; however, the histone ubiquitination remains highly unexplored under AKI settings.

2.4. Renin-angiotensin system in acute kidney injury

RAS is one of the crucial regulatory systems for blood pressure and fluid balance. As RAS has Janus's face, it is classified in its two major axes: conventional and non-conventional axis. The principal effector of its conventional axis, Ang II is released by local and systemic RAS activation and in renal vasoconstriction as well as inflammation and tubular damage led to AT1R activation (Kwon et al., 2003; Malek & Nematbakhsh, 2015; Ruiz-Ortega et al., 2003). On the other hand, the non-conventional or protective axis of RAS, which exerts its vasodilatative effects in various tissues (heart, kidney, lung, blood vessels) (da Silveira et al., 2010; Ocaranza & Jalil, 2012; Ruiz-Ortega et al., 2003). It is acknowledged that altered levels of RAS components exert important roles in several pathophysiological conditions (Kobori et al., 2007). Renin (also known as angiotensinogenase) is a key enzyme of the RAS i.e. selectively produced inside the juxtaglomerular apparatus in each nephron. Renin converts angiotensinogen (AGT) to Ang I, which is further hewed by ACE

into Ang II (Bernstein et al., 2001). Ang II (major product) mediates its physiological role via two receptors: AT1R and AT2R (Kanasaki et al., 2011). Ang II serves to be the major downstream molecule of the conventional axis of RAS, however, the substantial research done onto RAS has identified several other peptides that are equally significant.

Therefore, another axis of RAS referred to as the non-conventional RAS. ACE2 is the key enzyme of non-conventional RAS by cleaving Ang I and Ang II into Ang (1-9) and Ang (1-7), respectively. Both of these peptides exert opposite effects of Ang II (Bader, 2013; Xia & Lazartigues, 2010). Ang II is further cleaved into angiotensin III [Ang (III)] and angiotensin IV [Ang (IV)], that act via AT1R and AT4R. Ang IV regulates cognitive, learning memory, and regulates glucose uptake (Albiston et al., 2011; Braszko et al., 2006). A schematic representation of the RAS components is shown in ***figure 2.3***.

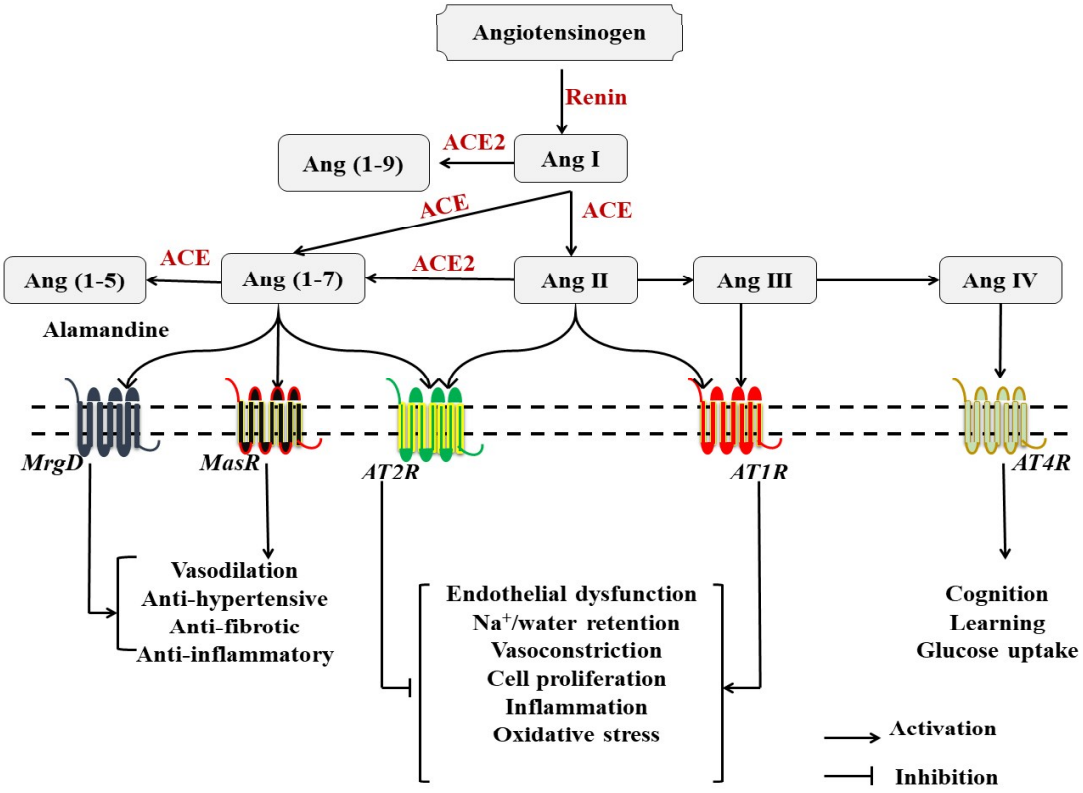

Figure 2.3 RAS components and their respective receptors

2.4.1. The pressor arm: the fiend in the renin-angiotensin system

AGT is the parent polypeptide from which all other angiotensin peptides are formed. Structurally, human AGT is made up of 485 amino acids, involving a 33 amino-acid signal

peptide. The 10 amino acid sequences on N-terminal are cleaved by renin into Ang I, which acts as the source for a range of active angiotensin peptides. AGT has been reported to play crucial roles in cardiovascular and renal disorders including AKI (Ba Aqeel et al., 2017; ElAlfy et al., 2018). One study conducted by a group of scientists has explored the role of activated protein C on the altered expressions of different components of RAS including AGT in lipopolysaccharide-induced AKI (Gupta et al., 2007). The pathogenesis involves endotoxemia, disturbed hemodynamics, and increased production of nitric oxide (NO) along with renal vasoconstriction. There were an increased protein and mRNA levels of AGT and ACE but suppressed ACE2 levels which were interestingly reversed by activated protein C treatment. There are numerous biomarkers reported for the early diagnosis of AKI (Alge et al., 2013; Bihorac et al., 2014; Herget-Rosenthal et al., 2004; Kashani et al., 2013; Mishra et al., 2003; Parikh et al., 2004; Portilla et al., 2008; Shao et al., 2014). Amongst all, urinary angiotensinogen (uAGT) has also joined the biomarkers' category that could serve in early diagnosis of AKI. It is highly stable in urine and helps in providing more appropriate information regarding RAS activity (Kobori et al., 2002). Further, a fine compilation of several studies carried out by Sheeba A et al. has represented AGT as a biomarker for AKI (Ba Aqeel et al., 2017). AGT is the parent polypeptide that generates other RAS peptides, therefore its significance as of urinary biomarker makes it more convenient in regulating the severity of AKI.

2.4.1.1. Role of renin in acute kidney injury

Renin, a specific endopeptidase, which is synthesized as an inactive proenzyme said to govern the rate-limiting step in the generation of RAS metabolites. The synthesis and secretion of renin are regulated by the sympathetic nervous system, blood pressure, extracellular fluid, and prostaglandins. It generally cleaves AGT to Ang I that increases blood pressure along with renal sodium retention (Persson, 2003; Wong, 2016). Preclinical studies depicted that renin binds to its receptor and activates mitogen-active protein kinase p44/42 and transforming growth factor-beta (TGF- β) led to increased kidney hypertrophy, and fibrosis (Wong, 2016). Moreover, aliskiren belongs to a novel class of renin inhibitors (Allikmets, 2007). Aliskiren has therapeutic potential in hypertension, diabetes, myocardial infarction, and CKD (Bavishi et al., 2016; Solomon et al., 2011). Apart from these studies, aliskiren also has protective effects in animal models of unilateral ureteral

obstruction and CKD (Choi et al., 2011; Lizakowski et al., 2012; Moriyama et al., 2012; Woo et al., 2013). Interestingly, it has also shown a promising effect in experimental and human AKI (Azizi & Menard, 2013; Hammad et al., 2013; Harel et al., 2012). Hammad *et al.* reported the effect of aliskiren before, after, and during AKI in an animal model of renal I/R (Hammad et al., 2013). Further, another study evaluated its role in AKI by electron microscopy and molecular studies (Ziypak et al., 2015). Aliskiren reduced the induction of iNOS, interleukin-1β (IL-1β), Ang II, and NF-kB. It decreased sCr and BUN levels along with improved morphological changes. Thus, the above-mentioned reports have proven the reno-protective role of aliskiren in AKI. Therefore, direct renin inhibition appears to be effective in attenuating AKI observed with clinical and preclinical conditions.

2.4.1.2. Role of angiotensin-converting enzyme in acute kidney injury

ACE is a di-carboxypeptidase enzyme cleaving 2 amino acids from the c-terminus of the inactive precursor Ang I to produce the vasoactive peptide Ang II (Corvol et al., 2004). ACE has two large homologous domains: N and C domains, which showed a difference in the binding properties for ACEi (Wei et al., 1992). Further, ACE has different genetic variants at plasma and tissue levels. These variants of ACE gene have been associated with various vulnerabilities to hypertension, diabetes, cardiovascular, and kidney diseases (Marre et al., 1994; Marre et al., 1997; Rigat et al., 1990; Sayed-Tabatabaei et al., 2006; Takahashi & Smithies, 2004). A strong link between ACE and disease exists for AKI (Pazoki-Toroudi et al., 2003; Vilander et al., 2015). Kidney ischemia has shown to alter the balance of the RAS axis (Mackie et al., 2001). Increased ACE expression contributes to the activation of numerous pathological pathways such as the release of ROS, oxidative stress, inflammation, fibrosis, and apoptosis which are attenuated by procuring ACEi. One report has proved the mechanistic approach of captopril sovereign to ATP-dependent potassium channels in curbing I/R renal injury (Habibey et al., 2008). Increased ACE levels are associated with elevated biochemical markers of excretory kidney function (cystatin C and creatinine) and intrarenal injury [interleukin-6 (IL-6), TGF-β, interleukin-10 (IL-10), Ang II] in I/R rats (Efrati et al., 2011). The ACEi, captopril reported attenuating kidney injury via suppressing inflammation, intrarenal NO release, and amassing of Ang II at the initial phase of reperfusion (Efrati et al., 2011). Apart from captopril's ACE inhibiting activity, it also possesses an anti-oxidant property via its thiol moiety (Fouad & Jresat,

2013). Thus, in a comparative study performed to treat kidney IR injury, it inhibits ROS and lipid peroxidation by reducing urea, creatinine and NGAL levels in the IR kidney (Kocak et al., 2016). Interestingly, ACE is set to play a major role in the conversion of the active forms of bradykinin and kallidin to their inactive forms. Whereas ACEi has shown beneficial effects (partly via kinins) in ischemic AKI (Chiang et al., 2006). However, there are conflicting reports for bradykinin use in I/R. Indeed, exogenous bradykinin can aggravate injury, while depletion in endogenous bradykinin led to the worsening of AKI (Chiang et al., 2006; Kakoki et al., 2007). Furthermore, bradykinin also exerts its action by increasing NO levels and phospholipase A2-derived products in ischemic AKI (Paller et al., 1992). Because ACEi is known to reduce glomerular perfusion pressure, clinical reports have shown controversial effects of ACEi on the progression of AKI in patients undergoing cardiac surgery (Meersch et al., 2017). So, it is difficult to conclude the role of ACEi in worsening or alleviating AKI and further studies are needed to clarify this contentious mechanism.

2.4.1.3. Role of angiotensin II in acute kidney injury

Ang II, an octapeptide hormone, is the active product of RAS generated by the cleavage of Ang I. It is produced systemically and locally through "renal" and "tissue" RAS, respectively. Ang II controls renal blood flow and helps in maintaining homeostasis (De Gasparo et al., 2000), and also exerts deleterious effects such as differentiation, proliferation, regeneration, oxidative stress (Kim et al., 2012; Lopez et al., 2003), and apoptosis (Zou et al., 2012). It acts as a heady intrarenal vasoconstrictor and GFR regulator, along with it stimulates hypertrophy and promotes mitogenesis in proximal tubule cells by binding to apical or basolateral receptors (Isaka, 2016; Isobe-Sasaki et al., 2017; Jha et al., 2016; Yacoub & Campbell, 2015). Furthermore, the different hemodynamic and tubular growth responses exerted by Ang II are supposed to be mediated via receptor; AT1R. So, the activation of RAS along with elevated Ang II levels emerged as the major risk factors for AKI (Kontogiannis & Burns, 1998; Yang et al., 2012).

A study by Alicia J. L *et al.* had revealed the differential actions of renal I/R injury on the intrarenal RAS. AKI was associated with a significant elevation of in renal levels of Ang II, while tissue Ang I or Ang (1-7) remained unchanged. The raised Ang II levels may be a direct consequence of induced cortical renin activity. These changes were short-lived

providing a mechanism for the decrease in Ang II-induced vasoconstriction following ischemia. In addition, Ang II also triggers aldosterone production in the adrenal gland, and its role has been evaluated for the development and progression of kidney injury (Chrysostomou et al., 2006; Zhou et al., 2004). Gupta, A. et al revealed the effect of activated protein C on local RAS in the lipopolysaccharide-induced endotoxemia model of AKI (Gupta et al., 2007). In this, lipopolysaccharide leads to the upregulation of deteriorative RAS components, including Ang II. Furthermore, they speculate on the decrease in Ang II synthesis, which may participate in improving kidney functions.

Apart from its conventional role in renal sodium and electrolyte transport, Ang II tends to increase kidney tissue injury independently of blood pressure and renal hemodynamics (Rafiq et al., 2011). Therefore, in some studies the effects of the mineralocorticoid antagonist spironolactone on Ang II and aldosterone levels were evaluated against AKI (Sanchez-Pozos et al., 2012); out of which one has focused on the administration of spironolactone before or after AKI and found that this intervention attenuates the development of CKD upon AKI (Barrera Chimal et al., 2013). However, CKD is a common problem in AKI survivors. A marked reduction of Ang II and aldosterone levels along with decreased inflammatory and fibrotic markers has been reported with spironolactone treatment even before or after ischemia, specifying the promising role of spironolactone as a preventive measure for AKI-induced CKD (Barrera Chimal et al., 2013).

2.4.1.4. Role of angiotensin II type 1 receptor in acute kidney injury

In the year 1991, the cloning of the AT1R was completed, which allowed the progression of research on the function of this receptor (Murphy et al., 1991; Sasaki et al., 1991). The AT1R is composed of 359 amino acids (40kDa) and belongs to the seven-membered G protein-coupled receptor superfamily (Higuchi et al., 2007). AT1R signaling contributes to blood pressure regulation, vasoconstriction, cardiac contractility, renal tubular sodium absorption, and cell proliferation. It also tends to effectuate detrimental effects by activating numerous pathological pathways such as inflammation, oxidative stress, endothelial dysfunction, cardiovascular and renal diseases (Griendling et al., 2000; Kim et al., 2017; Macia Heras et al., 2012; Scalia et al., 2011; Touyz & Schiffrin, 2000) ***(Figure 2.3)***. Indeed, the AT1R inhibitor losartan has anti-inflammatory activity by detaining TNF-α, IL-1β, IL-6, and avert leucocytes infiltration and thus granting renoprotection against

I/R injury (Molinas et al., 2009; Srisawat et al., 2015). Moreover, Mehrotra .P et al. confirmed the effect of a high salt diet and the role of AT1R activity on CD4 T cell activation and their latent effects on the development of CKD post-AKI (Mehrotra et al., 2015). In general, Ang II signals get intensified post-AKI and high salt diet amplifies Ang II-induced tissue damage (Basile et al., 2012). These authors found an elevated number of activated T-cells along with IL-17 expressions acting as essential mediators of inflammation and fibrosis. The association of activated T cells and AT1R activity in AKI progression to CKD is supported by De Miguel et al., that revealed the elevated intrarenal Ang II levels within renal lymphocytes compared to whole kidney tissue (De Miguel et al., 2010), signifying the influence of local rather than systemic RAS activity that actually might contribute in salt-induced lymphocyte activation. Losartan treatment increased renal blood flow, a process inhibiting pro-fibrotic factors such as TGF-β and post-AKI fibrosis (Mehrotra et al., 2015). Furthermore, one more interesting finding suggested the potential of AT1R antagonism in the prevention for transition of ischemia insults induced renal injury to CKD by suppressing different maladaptive mechanisms (Rodriguez Romo et al., 2016).

At three days post-AKI was accompanied by renal dysfunction, tubular atrophy, and inflammation, which almost recovered in losartan-treated rats. Rats kept for 9 months upon AKI developed CKD, which was characterized by proteinuria, kidney hypertrophy, glomerulosclerosis, tubular atrophy, and interstitial fibrosis. This process was associated with elevated expression levels of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1α (Rodriguez Romo et al., 2016). Hence, AT1R blockade prior to ischemia might help in averting AKI to CKD transition via ameliorating early renal blood flow recovery, reduced inflammation, and amplified HIF-1α activity (Figure 3). We can conclude that the induction and activity of the AT1R has a prominent role in the pathogenesis of AKI and the use of AT1R blockers has revealed their promising role in curbing long-term outcomes of AKI.

2.4.2. The depressor arm: the friend in the renin-angiotensin system

2.4.2.1. Role of angiotensin-converting enzyme 2 (Nature's ACEi) in acute kidney injury

ACE2 is a monocarboxypeptidase, the only identified homolog of ACE, and has a 42% sequence homology with the N- and C-terminal domains of somatic ACE (Crackower et

al., 2002; Donoghue et al., 2000; Donoghue et al., 2003; Turner et al., 2002). It is a negative regulator of RAS as it degrades Ang II to Ang (1-7) (Corvol et al., 2004). ACE2 is highly expressed within the kidneys in tubular epithelial cells, in glomerular epithelial cells, and in the renal vasculature. ACE2 activity has reported getting altered in numerous types of kidney injury (Bae et al., 2017; Lv et al., 2017; Ross & Nangaku, 2017). Recently, several studies revealed the role of ACE2 in different animal models of AKI. Gupta et al., have found the efficacy of activated protein C as an anti-inflammatory agent, which can hold back lipopolysaccharide-induced AKI by significantly upregulating the mRNA levels of ACE2 in renal tissue (Gupta et al., 2007).

Da Silveira et al. (da Silveira et al., 2010) examined the renal profile of ACE2, including Ang (1-7) and the MasR in renal I/R injury. Rats were subjected to left nephrectomy and ischemia (45 min) followed by reperfusion (2h or 4h) in the right kidney. Renal ACE2 activity was diminished along with reduced levels of Ang (1-7) and increased levels of MasR further illustrating the complexity and importance of ACE2-Ang (1-7)-MasR alliance in AKI. An altered ratio of ACE/ACE2 results could affect disease outcomes by escalating oxidative stress. Moreover, Yang *et al.* have demonstrated the effect of imbalanced ACE/ACE2 in tourniquet-induced kidney injury. Diminished ACE2 levels promulgate the disease along with increased free radicals and suppressed anti-oxidant levels (Yang et al., 2012). Recently, Soler et al. studied circulating ACE2 activity in kidney transplant patients and found that the enzymatic activity of ACE2 correlated with graft function, glycosylated hemoglobin, and liver function tests (Soler et al., 2012). Thus, serum ACE2 levels could be a non-invasive marker of the function of RAS in kidney transplant patients,ts. In addition, urinary ACE2 activity and ACE2 protein got augmented in transplant recipients, moreover the co-existence of diabetes has shown a major association with urinary ACE2 levels and thus served as a biomarker in such patients (Xiao et al., 2012). These findings might be helpful to exert a special importance for a variety of clinical relevance. Apart from the research on ACE2 regulation, some therapeutic regimens have also been studied in the AKI context.

Diminazene Aceturate (Dize): ACE2 activator

Malek *et al.* established the functional role of ACE2 using Dize in gender-based I/R kidney injury (Malek & Nematbakhsh, 2014). When given to male rats the ACE2 activator

markedly reduced markers of kidney and liver dysfunction, while no such effect was noted in female rats. DIZE could elicit antioxidant effects and increased nitrite levels in the male kidney (Malek & Nematbakhsh, 2014) however, biochemical and oxidative stress parameters did not conclude much about the action of DIZE. So, more studies are required to explore the mechanisms underlying these reno-protective effects of ACE2. Thus, these findings suggest that ACE2 has a potential role in the pathogenesis of AKI ***(Figure 2.4)***.

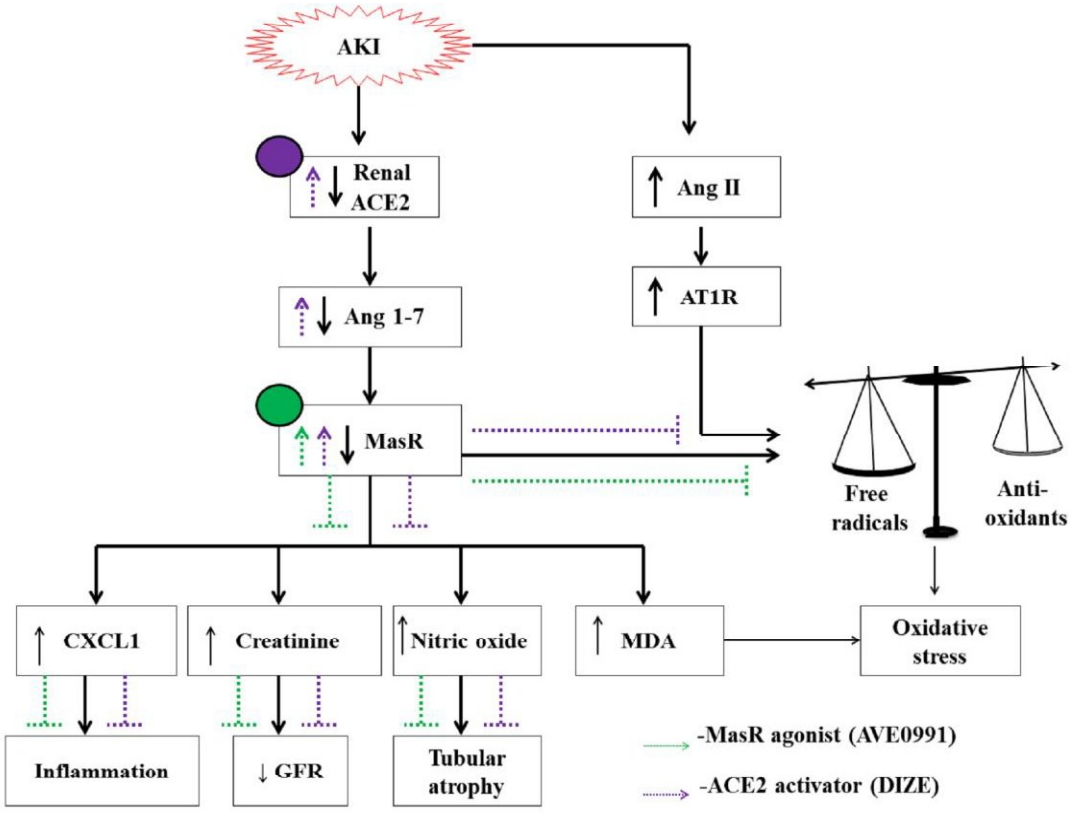

Figure 2.4 Role of non-conventional axis [ACE2/Ang (1-7)/MasR axis] in the progression of AKI.

2.4.2.2. Role of angiotensin (1-7) in acute kidney injury

Ang (1-7) is an active 7-amino acid peptide (Asp-Arg-Val-Tyr-Ile-His-Pro) generated by the hydrolysis of Ang II via ACE2 activity (Donoghue et al., 2000; Vickers et al., 2002). Ang (1-7) has reported to exert its action via MasR (Santos et al., 2003; Xu et al., 2008) and also act via AT2R (Gorelik et al., 1998). Though the ACE2/Ang (1-7)/MasR axis has emerged as a novel pathway in AKI, its molecular effects are still unclear. Ang (1-7) is

said to counter-regulates various harmful effects of Ang II by releasing the vasodilatory mediators NO, prostaglandin E2, and bradykinins (Santos et al., 2018). Studies have suggested the participation of Ang (1-7) in kidney inflammation and fibrosis (Mori et al., 2014; Santos et al., 2008; Varagic et al., 2014). To understand the interplay between Ang (1-7) and MasR, Esteban et al. employed animal models of unilateral ureteral obstruction and I/R injury. They delineated the effect of Ang (1-7) on inflammatory cascades via MasR. When MasR-deficient mice were infused with the Ang (1-7), they showed a decreased inflammatory response compared to wild type mice. The major findings of this study demonstrate a new avenue for other inflammatory diseases (Esteban et al., 2009). Furthermore, Da Silveira et al. explored the ACE2/MasR/Ang (1-7) axis in postischemic AKI. The upregulation of Ang (1-7) and MasR levels had checked by biochemical assays, immunohistochemical and western blot analysis. Elevated expressions of MasR and Ang(1-7) had confirmed their connection and prospective in the pathogenesis of AKI (da Silveira et al., 2010).

Peptide Ang (1-7) analogs mimic Ang (1-7) activity, e.g. CGEN-856 or the cyclic structural Ang (1-7) analog (Pancyte) and their activity have been tested *in vivo* for cardiovascular disorders such as cardiac arrhythmias and pulmonary hypertension (Machado-Silva et al., 2016; Savergnini et al., 2010). Due to poor oral bioavailability, Ang (1-7) has been encapsulated with hydroxyl-propyl β-cyclodextrin, which can attenuate cardiac complications and improve type 2 diabetes in a rat model (Bertagnolli et al., 2014; Santos et al., 2014). Although, these available analogs have not yet been explored in AKI. Moreover, Ang (1-7) plays a crucial role in potentiating blood cell reconstitution after toxic myelosuppression. (Ellefson et al., 2004; Heringer-Walther et al., 2009). With such reports effect of Ang (1-7) on HLA-I+ and CD34+ cells (progenitor cells) was evaluated where it arouses the proliferation and differentiation of stem cells. The progenitor cells are defined by their limited self-renewal ability and give rise to a specific type of mature cell. Hence, they said to play a significant role in tissue regeneration (Reule & Gupta, 2011). Due to its regenerative ability, they were also explored in kidney tissue where they have given significant outcomes (Reule & Gupta, 2011). Ang (1-7) could be explored as a stimulator of progenitor cell proliferation and differentiation also in kidney regeneration upon AKI as

previously shown for other drugs. Thus, a lot of work is yet to be done to fully explore the role of Ang (1-7) in AKI.

2.4.2.3. Role of Angiotensin (1-9) in acute kidney injury

Ang (1-9) is a crucial member of the RAS having nine amino acids in its structure (Basso & Terragno, 2001). Ang (1-9) is produced from Ang I by the action of ACE2, carboxypeptidase A or cathepsin A (Jackman et al., 2002; Kokkonen et al., 1997). It is reported to be widely expressed in platelets, heart, and kidney (Gonzalez et al., 2018; Kramkowski et al., 2010; McKinney et al., 2014; McKinney et al., 2015; Ocaranza & Jalil, 2012). Systemic levels of Ang (1-9) are detectable at 2-6 fmol/ml in healthy individuals, but these levels increase during disease conditions (Ocaranza et al., 2006). Moreover, Ang (1-9) exerts its protective effects in experimental hypertension and cardiovascular remodeling (Ocaranza et al., 2014). Recently, one study performed using deoxycorticosterone acetate hypertensive rat model, to demonstrate the anti-inflammatory activity of Ang (1-9) via AT2R on cardiac and kidney inflammation and fibrosis (Gonzalez et al., 2018). Intravenous administration of Ang (1-9) has lessened macrophage infiltration in kidney parenchyma and diminished tubular-interstitial fibrosis by attenuating collagen deposition. Henceforth, this explanation prompted the conclusion that Ang (1-9) has a kidney protective effect and might be evaluated under AKI settings.

2.4.2.4. Role of alamandine in acute kidney injury

Recently, alamandine [Ala1-Ang-(1-7)] was revealed as another previously unknown endogenous peptide of RAS. This heptapeptide is produced either by decarboxylation of the N-terminal aspartate residue of Ang (1-7) or ACE2-mediated hydrolysis of Ang II (Hrenak et al., 2016). Although alamandine and Ang (1-7) act via different receptors, their biological actions are quite similar (Leao et al., 2016). Alike Ang (1-7), alamandine binds specifically to a G-protein coupled receptor, i.e. MrgD (Mas-related genes) (Etelvino et al., 2014; Tetzner et al., 2016; Villela et al., 2014) ***(Figure 2.3)***. Lautner *et al*. conducted a preclinical study to characterize alamandine and its function in rat heart (Lautner et al., 2013). They revealed that alamandine is produced from the heart and also present in the blood. Further, the study was continued to evaluate its efficacy in hypertensive and fibrotic heart rat models, where it produces a long term antihypertensive and antifibrotic effects in

rat heart (Lautner et al., 2013). Thus, it is an interesting area of further research and could be explored in the AKI condition.

2.4.2.5. Role of Mas receptor in acute kidney injury

The Mas oncogene acts as a receptor for Ang (1-7) (Santos et al., 2008; Santos et al., 2003). Pharmacologically, Mas belongs to the class of G-protein-coupled receptors. It is made of 325 amino-acids and is highly expressed in brain and testis and less in other tissues, such as lung, spleen, heart, kidney, tongue, and skeletal muscle (Metzger et al., 1995; Villar & Pedersen, 1994). In the kidney tissue, the major pathway of Mas signaling is the ACE2/Ang (1-7)/Mas axis. Promising approaches to exploit MasR stimulation include the development of peptide analogs or a non-peptide Mas receptor agonists in order to mimic Ang (1-7) activity (Bader et al., 2012). So far, MasR remains to be explored in the AKI context. Recently, a study evaluated the activity of MasR along with other RAS peptides in a model of postischemic AKI. Interestingly, MasR expression was found elevated, possibly due to the activation of defensive pathways coping with the kidney insult (da Silveira et al., 2010). Moreover, a study conducted on MasR gene knockout mice using AVE0991, a MasR agonist verified its reno-protective effect in I/R injury. It attenuated glomerular and tubulointerstitial damage and improved kidney injury in association with decreased leukocyte infiltration and chemokine (CXCL1) production (Barroso et al., 2012). Reversal of all histopathological and biochemical parameters has further proven the reno-protective activity of MasR. Indeed, MasR signaling suppresses numerous oxidative stress factors and inflammatory cascades ***(Figure 2.4)***. Overall enhancement of MasR stimulation increased endogenous Ang (1-7) levels by ACE2 activation. Thus, this approach actually leads to the simultaneous upsurge in Ang II deprivation along with the diminution of AT1R stimulation. Furthermore, this novel target and its related therapeutic strategies support the concept that the ACE2/Ang (1-7)/MasR axis contributes to renal I/R injury although the precise nature of signaling pathways awaits further investigation.

2.4.2.6. Role of angiotensin II type 2 receptors in acute kidney injury

The AT2R is a G-protein coupled receptors coupled to Gαi2 and Gαi3 proteins (Berry et al., 2001). The expression of the AT2R declines during development and is found only in some organs after birth but is overexpressed in pathological states (De Gasparo et al., 2000). Structurally, human, rat, and mouse AT2R encodes for a 363 amino acid protein.

The AT2R possesses five possible N-glycosylation sites, which impart for different molecular weights (68-113 kDa). The presence of two characteristic disulfide bonds present in the AT2R structure differs from the respective AT1R (Karnik et al., 2015). In addition to it, R. Carey presented an in-depth work on RAS (especially AT2R) (Abadir et al., 2012; Carey, 2005; Carey, 2017a). He introduced a compiled report on the role of AT2R in maintaining blood pressure and kidney function (Carey, 2017b). AT2R mediates its action by inducing the release of kinins (bradykinin, kallikrein), cGMP, and NO levels (De Luca Jr et al., 2013) which facilitates the natriuresis and lowering BP along with vasodilatory, anti-inflammatory, anti-oxidative stress responses (offsets the actions of AT1R) (Castoldi et al., 2014; Matavelli et al., 2011; Rompe et al., 2010; Villela et al., 2015) ***(Figure 2.3)***. It is now widely recognized that AT2R has opposite actions compared to AT1R. In the cardiovascular system, AT2R has also been reported to exert its role in cardioprotection via offsetting various effects of AT1R (Miura et al., 2010). Moreover, the AT2R activation results in the equipoise of blood pressure elevated by AT1R signaling (Ichiki et al., 1995). In cardiomyocytes; the activation of AT2R leads to inhibit the autophagy signaling referred by AT1R (Porrello et al., 2009). Apart from it, AT2R also exerts its pivotal role in insulin signaling (Karpe et al., 2012; Ohshima et al., 2012; Yvan-Charvet et al., 2005). Recently, Munoz and colleagues have identified that the inhibition of AT2R with PD123319 (AT2R antagonist) has increased adiponectin, MCP-1, TNF-α, and another cytokine levels in adipose tissue (Munoz et al., 2017). Although the importance and efficacy of this receptor are not much explored in the pathophysiology of AKI, data from few studies are presented in this section. Folic acid nephropathy is one of the typical models of toxic AKI. Folic acid-induced kidney injury elevated the mRNA levels of c-myc (transcription factor) and Bcl2 family proteins which are reported as cell death markers in tubular epithelium (Ortiz et al., 2000) and further results in decreased Bcl2/Bax ratio that favored cell death. Further, Ruiz-Ortega *et al.* observed that folic acid nephropathy was characterized by AT2R overexpression and apoptosis in tubular cells (Ruiz-Ortega et al., 2003). Thus, AT2R re-expression along with AT1R suggests the crucial role of this receptor in AKI. Initially, peptide AT2R agonists such as CGP42112A or LP2 were discovered but possess no oral bioavailability and low specificity for AT2R.

Compound 21: AT2R agonist

In the year 2004, researchers have succeeded to found a novel AT2R agonist named C21. C21 found to be orally and systemically active (Steckelings et al., 2012), having 4-6 h of plasma half-life. C21 has a C-terminal pentapeptide structure similar to Ang II but lacks AT1R affinity and was evaluated in human embryonic kidney cells to show 4000-fold selectivity towards AT2R (Wan et al., 2004). By virtue of its vast biological activities such as anti-oxidant, anti-inflammatory, anti-fibrotic, anti-apoptotic, and anti-hypertensive properties, C21 exerted beneficial effect in cardiac disorders like heart failure and myocardial infarction; neurological disorders like ischemic stroke (Dai et al., 2016; Joseph et al., 2014; McCarthy et al., 2014; Pandey & Gaikwad, 2017b). In 2015, C21 has been approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA) against pulmonary fibrosis, a rare disease that entitled the C21 as an orphan drug category (Bruce et al., 2015). Vicore pharma, the company which is taking care of C21 based clinical trials, affirmatively announced the no serious side effects of C21 extended the dose from 0.3–100 mg, being given to healthy volunteers (https://mb.cision.com/Main/15668/2898035/1101868.pdf). Under physiological conditions, the adult kidney shows a substantial AT2R expression in renal vessels, glomeruli, and the kidney tubules, which is reported to be augmented under dietary sodium depletion, ischemia, or tissue repair process (Namsolleck et al., 2014). Recently, C21 showed a renoprotective effect under T2D-induced diabetic nephropathy (Pandey & Gaikwad, 2017b) as evidenced by reducing caspase-mediated apoptosis and NF-κB mediated inflammation. Interestingly, in the last week of March 2020, Vicore Pharma given a clinical trial application for C21 against pulmonary fibrosis. Due to its indirect relation with ACE2, C21 has been received approval from UK authority for phase 2 trial against COVID-19 (EudraCT 2017-004923-63). SARS CoV-2 virus enters the cell utilizing the enzyme ACE2 and inactivate it. It further causes an imbalance between RAS, resulting in acute lung injury. Vicore pharma utilized the idea of ACE2 which serves as the natural ligand of AT2R. As C21 acts directly on AT2R, it could reduce the inflammatory mediators and evade the way of virus debilitates the system.

2.5. Distant organ dysfunction associated with acute kidney injury

Despite the existence of renal replacement therapy, AKI is still connected with high mortality and morbidity. In human beings, it seems hard to determine whether AKI is a cause or consequence of surplus morbidity. In animal models, though, it is increasingly evident that AKI persuades distant organ dysfunction (Grams & Rabb, 2012). Recognized pathways comprise the induction of remote oxidative stress, inflammatory cascades, apoptosis, and distinctive molecular expression. Precisely, mounting evidence links renal injury as an instigator and multiplier of hepatic, cardiac, and neurologic dysfunction (Lee et al., 2018). Proper identification of these pathways will be essential in emerging targeted therapies to improve outcomes in AKI.

2.5.1. Liver dysfunction associated with acute kidney injury

Even though connotations between acute liver injury and AKI and the response patterns of both organs to injury are well reported (Lazzeri et al., 2019) the molecular mechanisms and pathogenic pathways under this association remain to be studied (Bucsics & Krones, 2017). Experimental studies revealed that hepatic ischemia/reperfusion injury is associated with increased serum levels of kidney injury molecule-1, neutrophil gelatinase-associated lipocalin, and increased renal cortical malondialdehyde (MDA) and heme-oxygenase-1 levels (Sun et al., 2017; Zager et al., 2014). Yet, hepatic ischemia/reperfusion injury-induced azotemia does not signify intrinsic renal injury. In spite of clinical manifestations for acute liver injury precipitates AKI, rather AKI imparts to predispose liver injury. Hospitalized intensive care unit patients with AKI acquire liver dysfunction (Lee et al., 2018). Various clinical investigations have noticed the development of liver dysfunction in patients with AKI who suffered alterations in protein synthesis, metabolic pathways of protein, lipid and drugs, and accumulation of cystatin C and cytokines IL18, IL-1β and IL-6 (Blanco et al., 2019; Makris & Spanou, 2016; Vilay et al., 2008).

During an AKI episode, not only renal drug clearance but non-renal drug metabolism is affected, which predisposes to drug overdosing and toxicity (Blanco et al., 2019). AKI alters hepatic drug metabolism via uremic toxins (creatinine, urea), inflammatory cytokines (IL-1β, IL-6, IL-18, interferons, TNF-α), and chemokines (Lane et al., 2013; Lea-Henry et

al., 2018; Philips et al., 2014). Therefore, it has been recommended that drug dosing is carefully monitored in patients with AKI who are highly vulnerable to liver dysfunction.

2.5.1.1. Role of inflammation and apoptosis

The kidney-liver crosstalk in AKI involves systemic inflammation and regulated cell death (Golab et al., 2009). Experimental AKI studies revealed that in AKI the following genes are upregulated in the liver: interleukin-17A (IL-17A), IL-6, TNF-α, keratinocyte chemoattractant, IL-10, intercellular adhesion molecule 1 and monocyte chemoattractant protein-1 (MCP-1) (Golab et al., 2009; Park et al., 2011; Park et al., 2012). TUNEL positivity or presence of for activated caspase-3 confirms the manifestation of cell death inside the liver. Renal ischemia/reperfusion injury causes an abrupt increase in serum and kidney cytokine levels (chemokine (C-X-C motif) ligand 1, MCP-1, IL-10, IL-1β, IL-5) (Andres-Hernando et al., 2012; Holderied et al., 2020). Increased kidney cytokine production and decreased clearance severely both impact on the cytokine production inside the liver (IL-6, IL-10) (Andres-Hernando et al., 2012). Recently, Nakazawa, *et al*. demonstrated the effect of ischemic AKI-induced multiple organ dysfunction (Nakazawa et al., 2017). Here, bilateral ischemic AKI causes tubular necrosis, neutrophil extracellular trap formation, and cytokine production inside the kidney, further stimulate the neutrophil infiltration and cell death (TUNEL positivity) in liver tissue (Nakazawa et al., 2017).

2.5.1.2. Role of oxidative stress and reactive oxygen species

Park and Golab revealed that experimental AKI also leads to the production of free radicals in hepatocytes (Golab et al., 2009; Park et al., 2011). Besides, elevated MDA and suppressed levels of superoxide dismutase, catalase activity, and reduced glutathione (GSH) were also observed in the liver tissue of AKI animals (Golab et al., 2009; Park et al., 2011). In the bilateral renal ischemia rat model, antioxidant glutathione significantly reduced MDA levels along with improved liver histology (Golab et al., 2009). Fadillioglu, *et al.* upon studying the combined effect of renal ischemia/reperfusion injury and STZ-induced diabetes on the hepatic injury, confirmed the speculation about the probable synergy between hyperglycemia and AKI in exacerbating secondary liver damage. The hepatic levels of MDA, xanthine oxidase, protein carbonyl groups, myeloperoxidase, and nitric oxide were increased in diabetic and non-diabetic ischemic groups (Fadillioglu et al.,

2008). Postconditioning (POC), an intermittent disruption of blood flow at the starting of reperfusion has been studied on hepatic dysfunction after AKI episode (Seifi et al., 2014). In this study, Seifi, *et al.* demonstrated that POC has successfully decreased MDA and increased the superoxide dismutase activity in the liver tissue compared to the renal ischemia/reperfusion group, suggested the beneficial effect of POC on liver dysfunction (Seifi et al., 2014).

2.5.1.3. Role of metabolic acidosis

Metabolic acidosis is common in critically ill patients (Sun et al., 2019). The etiology of metabolic acidosis involves ketoacidosis, lactic acidosis, abrupt volume expansion, and kidney failure. In AKI, the development of metabolic acidosis is associated with increased mortality (Sun et al., 2019). Acidosis reduces renal blood flow in healthy volunteers and contributes to renal inflammation (Peppicelli et al., 2013). Apart from kidney function, metabolic acidosis alters hepatic function via resistance to insulin and growth hormone, as well as suppression of hepatic synthesis of insulin-like growth factor-1 (IGF-1) and growth hormone receptor expression (Challa et al., 1993). A contributor to metabolic acidosis is lactate production, which is excreted through the liver and kidney under normal conditions. In metformin-related AKI, reduced renal lactate clearance activates hepatic lactate uptake by augmenting the hepatic activity of phosphoenolpyruvate carboxykinase (Arroyo et al., 2011). In an experimental sta udy, metabolic acidosis showed higher mortality in rats, with marked decline in blood pH, plasma bicarbonate followed by drastic decline in GFR (da Fonseca Magalhães et al., 2016). Metabolic acidosis significantly elevated the NF-κB expression, which persisted even after the protection provided by HO-1. Though, the impact of sodium bicarbonate on the progression of severe metabolic acidosis with moderate or severe AKI is currently being evaluated in a clinical trial (NCT04010630).

2.5.1.4. Modulation in liver enzymes

In AKI-induced liver dysfunction, the severity of AKI determines the degree of alteration of liver-specific enzymes (Bakker et al., 2015). In unilateral AKI mice, elevated levels of AST, ALT, and bilirubin were shown along with augmented neutrophil infiltration (Golab et al., 2009). Seifi, *et al*. evaluated liver function parameters upon renal ischemic POC in ischemic rats and reported a marked increase in serum ALT and AST levels compared to non-ischemic rats (Seifi et al., 2014). Ischemia/reperfusion in diabetic rats causes

hepatic dysfunction by concomitant AKI and hyperglycemia, indicated by the higher ALT and AST levels in the diabetic ischemia/reperfusion groups (Fadillioglu et al., 2008). ***Figure 2.5*** summarizes the factors that contribute to AKI-induced liver dysfunction.

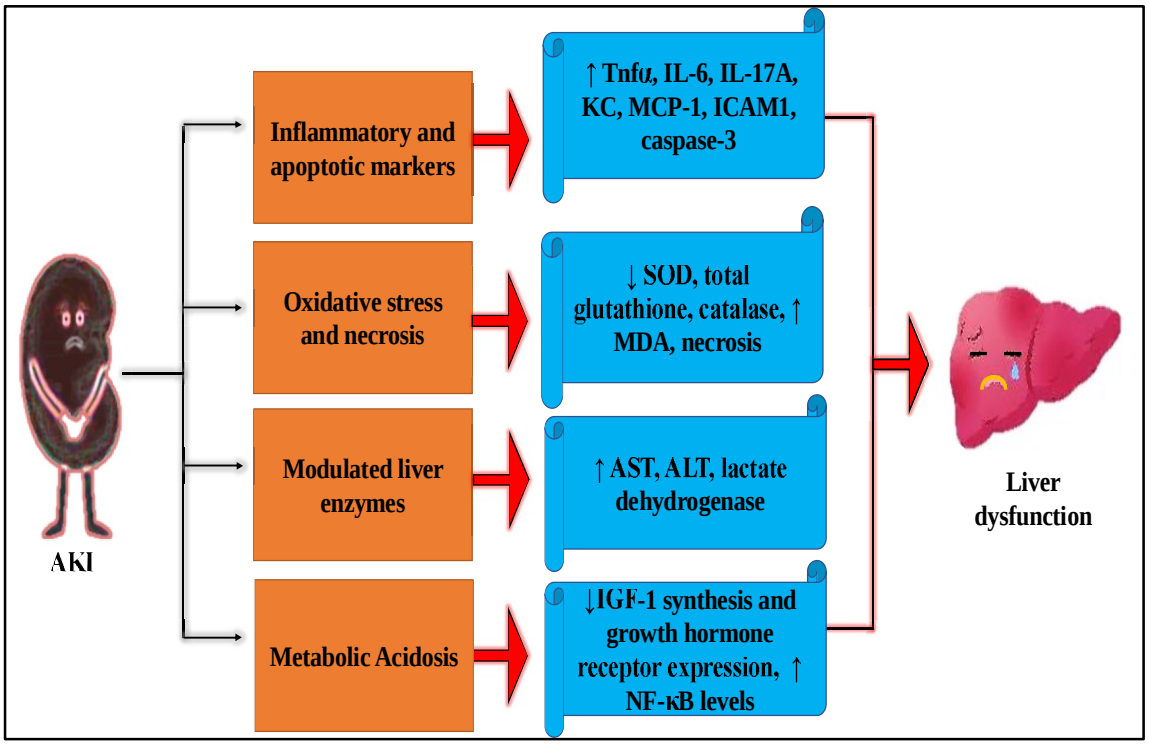

Figure 2.5 Pathophysiology of AKI-induced liver dysfunction.

AKI prompts liver dysfunction through several pathophysiological mechanisms, involving the accumulation of oxidants, altered liver enzymes level, inflammatory molecules, activation of apoptotic signaling. ***Note:*** *Tnfα, tumor necrosis factor-α; IL-6, interleukin-6; IL-17A, interleukin-17A; KC, keratinocyte chemoattractant; MCP-1, monocyte chemoattractant protein-1; ICAM1, intercellular adhesion molecule 1; SOD, superoxide dismutase; MDA, malondialdehyde; AST, aspartate aminotransferase; ALT, alanine transaminase; IGF-1, insulin-like growth factor-1.*

2.5.1.5. Therapeutic approaches to limit liver injury associated with acute kidney injury

Organ failure together with remote-organ system dysfunction contributes to the high overall mortality rate (Lee et al., 2018). In critically ill AKI settings, dialysis alone has not effectively diminished mortality. Golab, *et al*. found that administration of reduced glutathione markedly ameliorates the morphological and enzymatic alterations in hepatic tissue after AKI (Golab et al., 2009). Treatment with melatonin in ischemia/reperfusion-induced liver dysfunction rats has shown promising anti-oxidant effects by decreasing MDA, nitric oxide, and myeloperoxidase activity (Fadillioglu et al., 2008). Natural

products such as thymoquinone (an active constituent of *Nigella sativa* seeds) have been tested in AKI-induced liver injury, revealing its anti-oxidant and anti-inflammatory properties that significantly ameliorated kidney and liver function (Awad et al., 2011). Besides, thymoquinone also decreased the gene expression of CYP3A1 and spermidine/spermine-N1-acetyltransferase in the liver. Administration of vitamin E normalized GSH activity while attenuating the increased ALT and AST levels (Khastar, 2015). Isoflurane has been reported to have beneficial effects on ischemic and non-ischemic AKI-induced hepatic and intestinal dysfunction by activating the sphingosine kinase 1/sphingosine-1-phosphate pathway (Kim et al., 2010) ***(Table 2.3)***. Therefore, the validation of these potential treatments in clinical trials is needed.

Table 2.3 Potential therapeutic targets for AKI-induced liver damage.

AKI model	**Species**	**Treatment regimen**	**Important findings**	**Ref.**
IRI, BNx	Rat	Reduced glutathione	Improved structural and enzymatic liver activity	(Golab et al., 2009)
IRI	Rat	Melatonin	Reduced MDA, protein carbonyl, nitric oxide levels, Suppressed xanthine oxidase, and myeloperoxidase activity	(Fadillioglu et al., 2008)
IRI	Rat	Thymoquinone	Decreased liver-specific oxidative stress markers.	(Awad et al., 2011)
IRI	Mice	Vitamin E	Decreased ALT and AST levels, balanced oxidant-anti-oxidant levels	(Khastar, 2015)
IRI	Mice	Isoflurane	Diminished liver-specific enzymes (ALT, AST), reduction in hepatic inflammatory cytokines via the induction of AK1 in small-intestine	(Kim et al., 2010)

Note: *This table illustrates different treatment regimens demonstrated in AKI animal models. IRI-Ischemia/Reperfusion Injury; BNx- Bilateral nephrectomy.*

2.5.2. Brain dysfunction associated with acute kidney injury

2.5.2.1. Kidney-brain interactions: Clinical studies

AKI-induced uremic toxins are known to precipitate neurological dysfunctions, like irritability, attention deficit, hyperreflexia, mental retardation, seizures, and even death (Nongnuch et al., 2014). The degree of neurologic impairment is more closely linked with the severity index of kidney function decline, and not the levels of azotemia itself (Fraser & Arieff, 1988). Thus, uremia is responsible for the severity index in AKI in comparison to CKD patients (Burn & Bates, 1998). Numerous clinical studies claimed the long-term neurologic effects of AKI. A cohort study displayed the data of the elder group population affected with dialysis-requiring AKI. These patients showed higher chances of dementia incidence, regardless of adjusting other dementia-related risk factors (Guerra et al., 2012). One multicentered national study showed that patients with dialysis-requiring AKI were at greater risk of stroke in comparison to the control group, even after adjusting all other parameters, such as progressive CKD and diabetes (Wu et al., 2014). Siew et al conducted one prospective cohort study on 466 ICU patients with respiratory failure and/or shock. Stage 2 and 3 of AKI reportedly cause delirium in patients. Stage 3 AKI patients significantly suffered from delirium and coma. Whereas renal replacement therapy reduces this index by regulating the BUN and sCr levels. (Siew et al., 2017). In another nationwide cohort population study including 12-year follow-up period, AKI has contributed to the higher incidence of dementia (1.88-fold increased risk) even after adjustment of other factors, including age, sex, and other comorbidities (diabetes, cardiovascular, neurological and hepatic disorders) (Tsai et al., 2017). Therefore, careful monitoring and quick treatment for the emergency condition might reduce the chances of AKI associated neurological impairments in ICU patients.

2.5.2.2. Kidney-brain interactions: Animal studies

The pathophysiology of AKI-associated neurologic complications, involving the accumulation of neurotoxic metabolites that disturbs the integrity of blood-brain barrier and imparity in cellular water transport (Lu et al., 2015; Tanaka & Okusa, 2020). The bilateral renal IRI rat model exhibited severely declined renal functions along with the impaired locomotor activity. The dopamine turnover in the striatum, mesencephalon, and hypothalamus tissues dropped significantly, clarify the involvement of reduced dopamine

in the impaired locomotor activity (Adachi et al., 2001). In the experimental mice model of renal IRI following 24 hr of reperfusion, there was an increase in neuronal pyknosis and the number of activated microglial cells in the hippocampal section (Liu et al., 2008). Cortex and corpus callosum portion showed significant astrocyte activation, along with behavioral alterations as evidenced by decreased locomotor activity. Brain samples collected from renal IRI mice also had augmented levels of inflammatory cytokines both in the cerebral cortex and the hippocampus. Moreover, increased expression of Toll-like receptor 4 (TLR4) in the hippocampus and striatum (Salama et al., 2013); similar results were seen with AKI-induced lung injury (Doi et al., 2014) and therefore signifying the important role of TLR4 in AK- induced distant organ dysfunction. Although, the pathomechanism of AKI-associated brain dysfunction is highly unclear. A study conducted by Liu et al. clearly mentioned the increase in neuronal pyknosis or astrocyte activation in renal IRI, which was not found with liver IRI, signifying that neurological alterations might be specific for AKI and not just related with general inflammation following any organ injury. After ischemic AKI, disruption of blood brain barrier (BBB) might lead to infiltration of inflammatory molecules, like cytokines and chemokines ***(Figure 2.6)***.

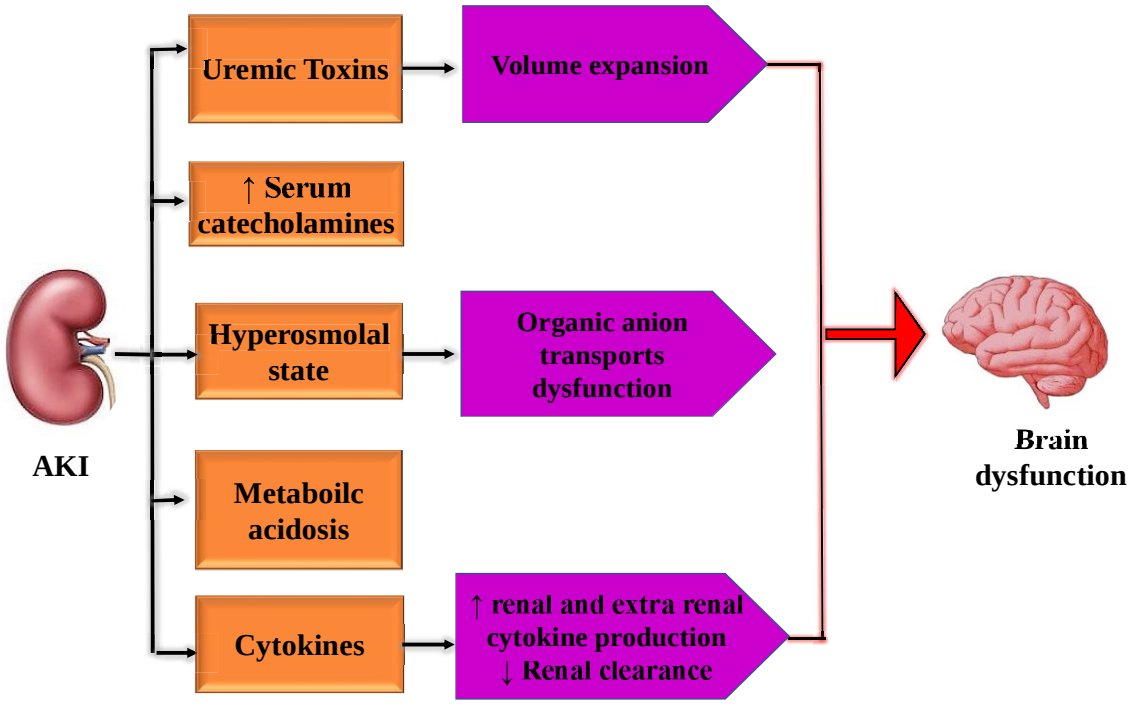

Figure 2.6 Pathophysiology of AKI-induced brain dysfunction.

Two studies demonstrated this concept with extravasation of albumin-bound Evans blue dye staining in comparison to sham groups (Liu et al., 2008; TSAO et al., 2001). Recently, Cao et al. have demonstrated the role of sympathetic reflex under renal IRI that interlinks the renal and cerebral RAS axis to elevated oxidative stress and progression of the injury. Interestingly, bilateral IRI promoted the intrarenal and cerebral, but not the systemic RAS. It promoted sympathetic activity in the renal and cerebral regions, and induced brain inflammatory cascade. Intracerebroventricular administration of losartan or tempol (anti-oxidant) decreased the renal ischemic score by 65% or 58%, respectively. Besides, selective renal afferent denervation with capsaicin or sympathetic tone by clonidine significantly attenuated the score by 42% or 52%, respectively. This study revealed a novel reno-cerebral sympathetic reflex pathway between ischemic kidney and the brain, contributing to renal IRI-induced brain inflammation.

2.5.3. Heart dysfunction associated with acute kidney injury

2.5.3.1. *Kidney-heart interactions: Clinical studies*

In the 1940s, the term "cardiorenal syndrome (CRS)" was proposed to explain the complex bidirectional crosstalk between heart and kidneys (Ronco, 2008). CRS is classified into five subtypes. Cardiac functions get acutely compromised by several bidirectional pathways activated during AKI, a description that has been termed as CRS- type 3 (Ronco et al., 2008), however, the pathomechanisms behind it are less understood (Bagshaw et al., 2013). Clinically, AKI patients mostly suffered from cardiac failure which remains the major cause of death (Jörres et al., 1999). The exact estimation of AKI patients showing cardiovascular events is unclear. In a multicenter cohort study, the incidences of cardiovascular failure observed around 60% of ICU patients having AKI (Liano et al., 1998). Another multinational cohort study revealed that the cardiovascular-related deaths remained the second most prominent cause of death in sepsis-induced AKI (Uchino et al., 2005).

Apart from short-term effects, AKI-induced long-term cardiovascular effects have also been observed. In a large population-based cohort study, AKI patient's data (from the year 1999 to 2008) was collected who recovered from *de novo* dialysis-requiring AKI, revealing the higher long-term risks for coronary outcomes, ultimately resulted in mortality irrespective of subsequent progression to CKD. It highlighted the AKI as the independent

variable for long-term cardiovascular risk (Wu et al., 2014). Another study on post-surgical AKI was reported to cause a 5-year risk for myocardial infarction and heart failure along with high mortality (Hansen et al., 2015). Nevertheless, further detailed clinical studies are required to elucidate the precise risk and impact of AKI-induced CRS.

2.5.3.2. Kidney-heart interactions: Animal studies

Numerous mechanisms have been demonstrated that may induce cardiac dysfunction during AKI. Conventional factors comprise fluid overload causes cardiac myocyte stretch and deteriorate myocardial performance (Wu et al., 2014). Accretion of uremic toxins and hyperkalemia contributes to cardiovascular toxicity and compromised coronary vasoreactivity, ultimately leads to myocardial ischemia (Singh et al., 2001). Several protein-bound uremic toxins exert a deleterious effect on our vital organs, like the kidney, heart, and blood vessels. Two most widely known protein-bound uremic toxins, including p-cresyl sulfate and indoxyl sulfate, revealed to induce vascular stiffness, calcification and ossification, and endothelial dysfunction (Lekawanvijit et al., 2016). Further, metabolic acidosis contributes to reduced myocardial contractility via alterations in β-receptor expression, change in intracellular calcium levels, electrolyte imbalances like hypo-/hyperkalemia cause life-threatening arrhythmias (Bagshaw et al., 2013). In addition to these classically known mechanisms, there are other pivotal factors with direct influence on heart tissue, including activation of the sympathetic nervous system (SNS) and the renin-angiotensin-aldosterone system (RAAS), and increased oxidant to the antioxidant ratio (Lee et al., 2018) ***(Figure 2.7)***.

One study conducted using a rat model of bilateral ischemic injury demonstrated a significant increase in protein and mRNA expressions of inflammatory molecules like TNF-α and IL-1, subsequent increase in myocardial apoptosis. This study also showed increased myeloperoxidase activity and abnormal echocardiographic findings, such as amplified left ventricular end-diastolic/end-systolic diameter, relaxation time, and diminished fractional shortening. It confirmed that renal ischemia, not uremia is itself enough to cause myocardial injury following AKI (Kelly, 2003). In the same study, TNF-α blocker expressively lessened cardiomyocyte apoptosis, suggesting the role of TNF-α in AKI-associated cardiac dysfunction. Considering the myocardial depressant effect of TNF-α, large multicenter trials conducted using anti-TNF-α therapies (etanercept) on congestive

heart failure, but the lack of clear evidence for clinical perspective, these treatments have been paused (Anker & Coats, 2002).

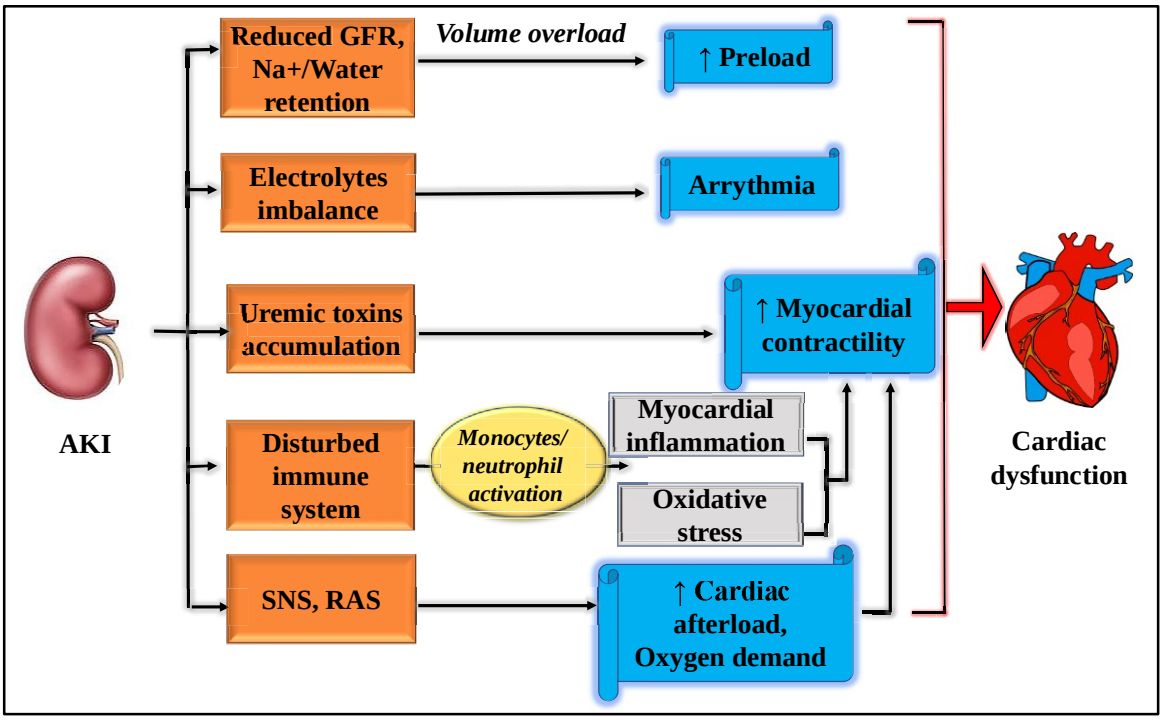

Figure 2.7 Pathophysiology of AKI-induced cardiac dysfunction.

During an ischemic injury, cardiac stroke volume, ejection fraction, and systolic pressure significantly reduced, with no alteration in end-systolic pressure-volume-relation. At the time of reperfusion, the abovementioned features initially upregulated followed by a gradual decrease. Increased CK-MB, BUN, creatinine levels assessed the myocardial and kidney injuries. In the same study, pretreatment with curcumin improved cardiac contractility and debilitated myocardial and renal injury via suppressing inflammatory response in the kidney and heart (Chen et al., 2013). Shen et al utilized the mouse AKI model to check the efficacy of endothelial progenitor cells (iEPCs) differentiated from human induced pluripotent stem (iPS) cells (Shen et al., 2018). iPS treatment increased the expression of the mature endothelial cell marker CD31 and substituted injured endothelial cells. It also improved the renal functioning, reducing the BUN and PCr levels. Cardiac dysfunction further evidenced by upregulating uremic toxins: indoxyl sulfate and IL-1β, inducing cardiac apoptosis. Administration of iEPC suppressed the proapoptotic protein (caspase-3) and overexpressed the anti-apoptotic protein Bcl-2 in the cardiac tissue of the AKI mice, probably by downregulating indoxyl sulfate and IL-1β. Another study

investigated the role of SNS and RAS on cardiac inflammatory signaling in a long-term ischemic renal injury mice model (Panico et al., 2019). Kidney injury was assessed by increased vimentin mRNA levels, however, augmented atrial natriuretic factor (ANF) showed the presence of cardiac lesions, effectively controlled by atenolol or enalapril treatment. Increased in mRNAs of TNF-a, IL-6, and IFN-γ further provided the cardiac inflammatory profile, which was significantly reversed by atenolol, losartan, or enalapril treatment. The SNS specific molecules, like β1-adrenoreceptors, adenylyl cyclase, PKA and noradrenaline markedly increased in cardiac tissue. Besides, renal ischemic injury upregulated cardiac renin and AT1R, which significantly regulated by losartan or enalapril treatment. Thus, cardiac inflammatory events induced by renal ischemic injury get triggered by the concurrent upregulation of SNS and RAS in the cardiac tissue. Recently, renal ischemic injury reported to downregulate the Nrf-2 expression, and antioxidant enzyme activities like superoxide dismutase, glutathione peroxidase, catalase. Treatment with naringin (flavanone) and trimetazidine, a well-known anti-ischemic drug effectively reduced the above-mentioned changes in the cardiac tissue, supposedly via the upregulation of Nrf-2 expression (Amini et al., 2019).